HAEMATOLOGY

LECTURES ON

HAEMATOLOGY

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PREFACE

The lectures published in this volume were delivered during a Symposium on Haematology held from 7 to 9 December 1959 at Cambridge under the auspices of the University Post Graduate Medical School The lectures were designed to provide a survey of current knowledge and opinion on a series of haematological topics of general interest and were directed to an audience of medical consultants senior registrars and research workers having haematology as one of their major laboratory or clinical interests The intention was not to present new and unpublished observations although a few such do appear in these pages but to provide informative reviews of expanding or controversial subjects compiled by lecturers who had themselves contributed to the advance of knowledge in their respective fields

The Post Graduate Medical School is most grateful for the helpful co operation of the contributors who accepted invitations to lecture and of the Cambridge University Press who have done their best to reduce the inevitable delay between delivery of the lectures and their publication

ACQUIRED HAEMOLYTIC ANAEMIA

J V DACIE

The subject of acquired haemolytic anaemia is a large one and in the space available it clearly will not be possible for me to review the whole field adequately I think the best thing I can do is to concentrate on a few of the more challenging aspects of the problem Thus after attempting to define the scope of the term acquired haemolytic anaemia I shall discuss in relation to the auto immune type first the auto antibodies secondly how the excessive haemolysis is brought about thirdly the effects of steroids and splenectomy on the course of the disease and whether the results of such treatment may be predicted and finally theories as to aetiology

First of all let me explain what I propose to include under the term acquired haemolytic anaemia There are of course many different kinds of haemolytic anaemia which are not congenital and which do not have an obvious hereditary basis but I think everybody will agree that the most important kind as well as the most thoroughly studied is that associated with the formation of antibodies active against the patient s own red cells—the so called auto immune type I shall in fact concentrate on this group and exclude from the discussion the secondary and symptomatic haemolytic anaemias which may accompany so many types of systemic disorder from disseminated carcinoma to severe renal or hepatic disease I shall also exclude from discussion cases of acquired haemolytic anaemia of unknown origin not associated with an underlying disease in which evidence of auto antibody formation cannot be demonstrated These latter cases form a fascinating and not unimportant group they are probably not homogeneous and their mechanism of haemolysis remains a baffling problem to which I can contribute little I shall however include in the discussion the rather remarkable acquired haemolytic anaemias associated with chronic lymphatic leukaemia and reticulosarcoma and allied disorders because these are often clearly of the auto immune type

Now as to the auto antibodies it is generally agreed that they conform to two types warm antibodies active at body temperature and cold antibodies seldom active at body temperature but strongly active usually at temperatures below 30–32 C The former are not often found in high concentrations in the patients sera the latter usually are present in very high concentrations and the antibody protein characteristically forms a distinct peak in the γ_1 position if the serum is subjected to paper electrophoresis Both types of antibody are adsorbed by the patient s own red

results The problem is nevertheless simplified if there is other evidence of auto antibody formation

I have studied several cases like this In one recent patient although the antiglobulin test was only weakly positive there appeared to be good evidence for the presence of a warm auto agglutinin which agglutinated normal red cells at body temperature moreover trypsinized cells were agglutinated to a high titre This patient clearly had an auto immune acquired haemolytic anaemia and as expected she responded to steroid therapy

Table 3 *Incidence of free antibody in patients sera demonstrable by the indirect antiglobulin test and by trypsinized red cells*

Test	Result	No of patients	Mean Hb level (g/100 ml)
AG T	– –	11 (3)	9 18
AG T	– +	9 (3)	8 71
AG T	+ +	20 (7)	6 27

The figures in brackets denote patients who had undergone splenectomy
The mean haemoglobin of the AG+ T+ series is sign ficantly lower than that of the AG– T– series (0 02 < P < 0 05)

It has to be admitted that there is no obvious correlation between the strength of the antiglobulin reaction as measured by the titre or the speed or intensity of agglutination and the severity of haemolysis *in vivo* However in individual patients the tests usually become less strongly positive if the patient recovers Again auto antibody free in the patient s serum is less easy to demonstrate in patients in remission than in those in whom there is active haemolysis This last point is illustrated in Table 3 This table summarizes observations on forty patients in whose sera antibodies active against cells of the patients own Rh genotype and ABO blood group were specially looked for Haemolysis was more intense as judged by low haemoglobin levels in patients in whom antibody could most readily be demonstrated in the serum

It is possible to do more with the antiglobulin test than to record merely whether it is positive or not and the speed of reaction and the serum titre The addition of human γ globulin in varying amounts to antiglobulin serum before it is used to agglutinate the patients corpuscles can be used to divide positive reactions into three groups (1) those in which very small amounts of γ globulin cause complete inhibition—the γ globulin type (2) those in which relatively large amounts of γ globulin cause no

cells—hence the use of the term 'auto antibody'—and they give rise to positive antiglobulin or Coombs's tests. In my experience, most auto-immune haemolytic anaemias are associated with the formation of antibodies of the warm sort. The relative distribution of the two types is shown in Tables 1 and 2.

Table 1. *Incidence of warm and cold types of acquired haemolytic anaemia*

Clinical type	Type of antibody	Patients: Males	Females	Total
Idiopathic	Warm	36	47	83
Idiopathic	Cold	4	13	17
Secondary (transient post virus pneumonia)	Cold	4	6	10
Secondary (chronic)	Warm	10	13	23
Secondary (chronic)	Cold	2	5	7
	Totals	56	84	140

Table 2. *Underlying disease in secondary auto immune haemolytic anaemia*

Type of antibody	Disease	No	
Warm	Reticulosarcoma, Lymphosarcoma	4	23
	Chronic lymphatic leukaemia	9	
	Other leukaemias	2	
	Lymphadenoma	1	
	D L E	5	
	Cirrhosis	2	
Cold	Reticulosarcoma, Lymphosarcoma	4	7
	Chronic lymphatic leukaemia	1	
	Myeloma	1	
	Lymphadenoma	1	

These figures are based on patients, or blood samples from patients, whom I have personally investigated in the last ten years or so. They are, of course, a selected series in the sense that some of the patients were referred because they were thought to be of special interest to me or to present particular problems. In particular, I believe that the relative incidence of the 'idiopathic' cold antibody cases is probably too high.

As already mentioned, a positive direct antiglobulin test is a characteristic feature of auto immune acquired haemolytic anaemia. Indeed, if the test is negative the patient cannot be included in the auto immune group. This is perhaps an arbitrary method of exclusion, but it is a useful one. A difficulty arises when the test gives doubtful or weakly positive

prove or disprove is that the non γ protein is protein adsorbed non specifically to cells damaged by antibodies

One of the most interesting recent discoveries in relation to the warm type of auto antibody in acquired haemolytic anaemia is the realization that in some of the patients the auto antibodies appear to be directed against an Rh antigen According to Hollander & Batschelet (1957) the reported incidence of the different Rh specific auto antibodies corresponds closely with the incidence in the population of the Rh antigens for example the fact that anti e has been most frequently reported can be explained according to Hollander & Batschelet *on the basis that the e antigen is the commonest* of the Rh antigens being present in all but 2–3% of red cell samples

Table 5 *Antiglobulin reactions in acquired haemolytic anaemia of the warm antibody type*

Type of reaction	Dilution of antiglobulin serum: *1 in 4*	*1 in 16*	*1 in 64*	*1 in 256*	*1 in 1024*	Control (saline)
γ globulin type						
Anti γ serum	+ + +	+ + +	+ + +	+ +	+	0
Anti non γ serum	0	0	0	0	0	0
Intermediate type						
Anti γ serum	+ + + +	+ + +	+ ±	0	0	0
Anti non γ serum	+ +	+	±	0	0	0
Non γ globulin type						
Anti γ serum	0	0	0	0	0	0
Anti non γ serum	+	±	0	0	0	0

The symbols + + + + + + + + + + and ± represent intensities of agglutination

Table 6 *Incidence of specific auto antibodies in thirty three patients with acquired haemolytic anaemia of the warm antibody type*

Non specific antibodies ≈ 23
Non specific + specific antibodies = 9
  Non specific + anti *e* (6)
  Non specific + anti e + anti c (2)
  Non specific + anti e + anti *D* (1)
Specific antibodies only
  Anti e + anti C = 1

Incidence of specific antibodies = 30%

Auto antibodies of definite specificity outside the Rh system have been reported (e g anti Jk van Loghem and van der Hart 1954 anti K Fluckiger Ricci & Usteri 1955) but clearly they are of the greatest rarity In my experience evidence of Rh specificity can be obtained only in a minority of cases (Table 6)

In most cases therefore the auto antibodies of acquired haemolytic

inhibition—the cold antibody or non γ globulin type and (3) those reactions of intermediate character in which the addition of small amounts of γ globulin causes appreciable but not complete inhibition It has now been established with fair certainty that the γ globulin type of reaction is given by red cells coated with γ-globulin antibodies—such as the Rh anti body anti D while the non-γ type or cold antibody type is due usually, if not always, to an interaction between complement adsorbed with the cold antibodies and the antiglobulin serum (Dacie Crookston & Christenson 1957)

Table 4 *Summary of results in twenty patients with auto immune haemo lytic anaemia of the warm antibody type using anti γ and anti non γ anti globulin sera*

Test	Result	No of patients
Anti γ Anti non γ	+ −	4
Anti γ Anti non γ	+ +	14
Anti γ Anti non γ	− +	2*

These two patients had formed complete auto agglutinins

The cause of the intermediate reactions is obscure The reactions suggest that the red cells are coated by other protein(s) in addition to γ globulin If to two samples of potent antiglobulin sera are added respectively γ globulin and globulins other than γ it is possible to obtain one reagent which reacts with cells which have adsorbed cold antibodies and not with cells which have adsorbed warm antibodies such as anti D and another reagent which reacts in exactly the opposite way

The results of using the two reagents with a series of red cell samples from cases of acquired haemolytic anaemia of the warm antibody type are shown in Table 4 Representative reactions of each type are shown in Table 5

The cause of the frequent reaction between the patients red cells and the anti non γ antiglobulin serum is obscure In several instances I have prepared an eluate from such cells and been able to show that eluted protein capable of coating fresh normal cells is a γ globulin I have no evidence therefore that the non γ protein is antibody protein Nor have I positive evidence that it is complement as has been shown to be the case with cold antibodies Cells reacting with both types of antiglobulin serum need testing with specific anti complement sera but I have not been able to do this An alternative and attractive hypothesis which is difficult to

So much for the antibodies How do they produce haemolysis *in vivo*? With warm antibodies the mechanism of haemolysis has been and still is to some extent a mystery However recent work particularly that in which ^{51}Cr tagged red cells have been used has produced useful information It is now thought when red cells are coated' with warm antibodies of the *incomplete variety (as they typically are in acquired haemolytic*

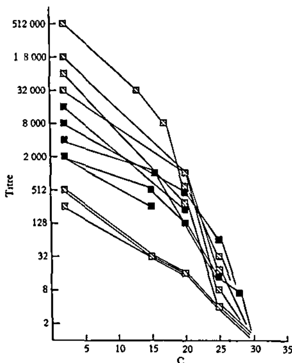


Fig 1 Titre and effect of temperature on agglutination by cold antibodies Black squares = sera from post virus pneumonia patients cross hatched squares = sera from patients with idiopathic acquired haemolytic anaemia of the cold antibody type

anaemia) that the coating of the cells with antibody leads when the cells are suspended in protein containing media to their adherence to each other and the formation of small agglutinates (Jandl & Castle 1956 Jandl Richardson Jones & Castle 1957) These small agglutinates are thought to be trapped in backwaters of the circulation particularly in the spleen The cells comprising the agglutinates probably become spherocytic and ultimately undergo lysis or are phagocytosed by reticulo endothelial cells

Work with Rh antibodies carried out by injecting a small volume of labelled D positive cells into subjects having anti D in their circulation

anaemia appear to have no definite specificity and to react at least to some extent with all human blood samples tested It is possible that such apparently 'non specific antibodies are really specifically directed against a public' antigen common to the vast majority of human red cells but this has not yet been proved, and in my view there is nothing strange in the idea that they react with a surface configuration common to *all* human red cells independent of the known blood group antigens

On the other hand it is possible that the antibodies, although basically anti Rh, have lost their specificity more or less completely Wiener, *Gordon & Gallop (1953) in fact postulated that the 'non specific' anti* bodies were directed against the 'nucleus of the Rh Hr substance' The situation is clearly complex for it seems that more than one component of antibody may be present at the same time Dacie & Cutbush (1954) demonstrated in three cases, for instance, that the 'non specific' component might be split up into two fractions while in one patient two 'non specific' unidentified fractions were present alongside anti D and anti e both of which were auto antibodies The matter is of practical as well as of theoretical importance for in the rare patients who develop a single anti e auto antibody R_2R_2 blood may be expected to survive normally *in vivo* with possible great benefit to the patient

Now I shall have to say something about cold auto antibodies These are quite distinct from the warm type Cold antibodies are macroglobulins ($S_{20,w}$ 16–19), whilst the warm types are probably $S_{20,w}$ 7 globulins Cold antibodies are powerful agglutinins but at the same time in contra distinction to warm auto antibodies they fix complement and are potentially haemolytic and as I have already mentioned the positive antiglobulin tests they produce are apparently due to their property of fixing complement (Dacie Crookston & Christenson, 1957) Cold antibodies react with at least the vast majority of human red cells (and also strongly with rabbit cells) It is however true that human red cells vary considerably in their sensitivity to the antibodies (Crookston Dacie & Rossi 1956) It has been suggested that this variation in sensitivity justifies the assumption that the antibodies and the corresponding antigens form a specific blood group system (Wiener Unger Cohen & Feldman 1956) but this has yet to be proved and until this is done I think it is reasonable to refer to them as non specific Finally the cold type of antibody may be present in rela tively enormous concentrations

This is illustrated in Fig 1 which also demonstrates the effect of tempera ture on the antibody s activity and in Fig 2 which shows an unusual sharply defined peak in the γ_1 region on electrophoresis the peak repre sents the cold antibody protein (Christenson & Dacie 1957)

scored as good in four patients fair in eight poor in six and doubtful in three It is possible that this series is weighted with seriously ill patients and that the results in an unselected series (if it were possible to obtain one) would be less gloomy It is nevertheless quite clear that splenectomy does not necessarily produce a good clinical result to say nothing of haematological cure Why is this?

There are probably several reasons for the failure of splenectomy First of all when overwhelming amounts of incomplete auto antibody are being formed it seems likely that haemolysis will take place to an important degree in organs other than the spleen particularly in the liver This is certainly true of Rh incompatible cells injected into recipients who have been either hyperimmunized against the injected cells or previously submitted to splenectomy (Jandl Richardson Jones & Castle 1957)

Secondly in some patients with acquired haemolytic anaemia the auto antibody seems capable of agglutinating the subjects red cells directly—the antibody acts as an in saline agglutinin Judged from experiments with in saline agglutinating isoantibodies such cells would be removed from the circulation more by the liver than by the spleen (Jandl Richardson Jones & Castle 1957 Cutbush & Mollison 1958)

Thirdly the spleen is likely in any case to be only a part source of the auto antibodies and it seems clear even when the operation is successful clinically that the success is due more to the removal of an organ important in haemolysis than the removal of an important source of the antibody

Is it possible to anticipate the success or failure of splenectomy? Chertkow & Dacie (1956) concluded before surface counting data on patients whose cells had been labelled with ^{51}Cr were available that it was not possible to predict the result on clinical or ordinary haematological grounds They found however that the operation seemed likely to be of least benefit in the most seriously ill patients just when in fact a good result was most urgently needed

To return to the question of prediction of the results of splenectomy The labelling of patients red cells with ^{51}Cr and the subsequent assessment of the radioactivity in the spleen and liver by surface counting using a scintillation counter seems likely to be of real help although the technique so far has been carried out only in a relatively small number of patients The results are what might have been expected In most patients an important amount of excess chromium accumulates in the spleen In some however accumulation in the liver is not negligible and in those patients in whom there is an obvious autohaemagglutination in the blood stream chromium accumulates in the liver to a marked extent (Jandl Greenberg Yonemoto & Castle 1956) Clearly the results of splenectomy will be of

has shown that under these circumstances the injected cells are removed from the circulation almost exclusively by the spleen (Jandl Richardson Jones & Castle, 1957 Hughes Jones Mollison & Veall 1957 Mollison & Hughes Jones 1958) This work fits in well with the earlier work on the function of the spleen in acquired haemolytic anaemia which I have already mentioned and is obviously relevant to the present problem bearing in mind the similarities between the auto antibodies of acquired haemolytic anaemia and Rh antibodies

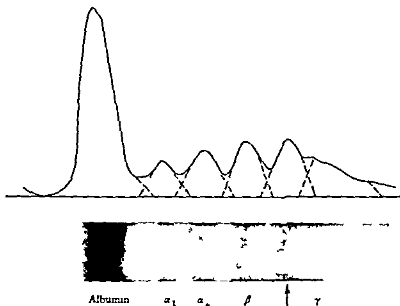


Fig Paper electrophoresis strip of the serum of a patient with the cold haemagglutinin syndrome The arrow points to the γ_1 globulin peak

The role of the spleen is of more than theoretical interest Micheli in 1911 is usually quoted as being the first to have carried out splenectomy in acquired haemolytic anaemia Be that as it may the problem as to whether the spleen should be removed in any particular case is certainly not a new one and we still have to think about it The experimental work on Rh antibodies that I have already mentioned which indicated the important role the spleen plays in filtering off cells coated with the anti bodies suggests that splenectomy should be of major benefit. In practice however although this is sometimes the case the operation not infrequently proves to be a failure

Chertkow & Dacie (1956) reviewed the results of splenectomy in twenty one patients with the idiopathic type of the disease The results were

scored as good in four patients fair in eight poor in six and doubtful in three It is possible that this series is weighted with seriously ill patients and that the results in an unselected series (if it were possible to obtain one) would be less gloomy It is nevertheless quite clear that splenectomy does not necessarily produce a good clinical result to say nothing of haematological cure Why is this?

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most value in patients in whom there is a major accumulation in the spleen and of least value when there is much in the liver also These latter are likely to belong to the most seriously ill group which Chertkow and Dacie found to fail to respond to splenectomy

The surface counting patterns in some of our own recent cases are illustrated in Fig 3

The pattern and mechanism of haemolysis in patients forming high thermal amplitude cold auto antibodies are different These patients suffer as a rule from only a slight to moderate degree of anaemia In winter

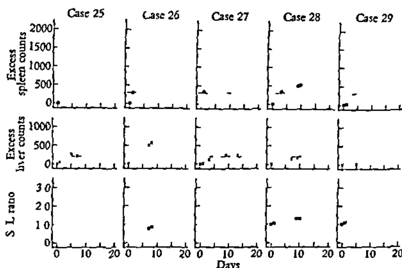


Fig 3 Surface counting patterns in five cases of auto immune acquired haemolytic anaemia of the warm antibody type after labelling the patients red cells with ^{51}Cr

however they become more anaemic and in extra cold weather they commonly suffer from major degrees of intravascular haemolysis leading to haemoglobinuria as well as from Raynaud s phenomena due to arrest of the circulation in skin vessels as a result of autohaemagglutination

The mechanism of haemolysis is probably twofold first it seems likely that agglutinated corpuscles when in high concentration as in the blood stream undergo haemolysis as the result of physical contact—this is the mechanical trauma hypothesis secondly haemolysis occurs in the blood stream as the result of complement lysis or following the removal of complement coated red cells by the liver and to a lesser extent by the spleen

From the patient s point of view it is the highest temperature at which the antibody will affect his own red cells that is important In anaemic

patients auto agglutination will usually be found to occur up to a temperature of 30–32 C If the antibody is inactive at 30 C the patient is likely to be hardly inconvenienced at all

Splenectomy has occasionally been carried out in patients with acquired haemolytic anaemia of the cold antibody type but dramatic improvement has not been recorded This is to be expected bearing in mind that the haemolysis appears to take place in the blood stream and in the liver rather

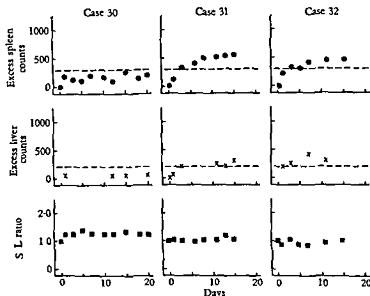


Fig 4 Surface-counting patterns in three cases of auto immune acquired haemolytic anaemia of the cold antibody type after labelling the patients red cells with Cr In case 30 there was no demonstrable accumulation of excess chromium in either liver or spleen in cases 31 and 32 excess chromium accumulated in both organs

than in the spleen predominantly The results of ^{51}Cr auto survival studies carried out on three patients with high titre cold antibodies in their blood are shown in Fig 4

I shall next refer briefly to the use of steroid hormones in the treatment of acquired haemolytic anaemia of the warm antibody type The first reports date from 1950 Now they are I think universally regarded as the first choice in the treatment of decompensated acquired haemolytic anaemia They appear to affect the patient s anaemia in several ways

First they probably diminish antibody formation secondly they appear to stimulate erythropoiesis as shown by a rise in the patient s reticulocyte count thirdly they possibly inhibit in an ill defined way antigen–antibody

interaction and erythrophagocytosis in the spleen and elsewhere The net result of these effects is usually clinical improvement in the patient and he often becomes able to achieve equilibrium between haemolysis and red cell formation at a tolerably high haemoglobin level Occasionally he is clinically cured, even so evidence of auto immunization usually persists

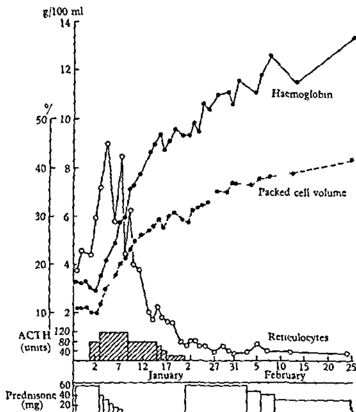


Fig 5 Effect of treating with prednisone and ACTH a patient with auto immune haemolytic anaemia of the warm antibody type The patient a woman aged 26 probably had disseminated lupus erythematosus as an underlying disease

and the antiglobulin test remains positive There is in fact no evidence that steroid hormones of any sort can *cure* acquired haemolytic anaemia of the auto antibody type

Despite its great benefits steroid treatment has some disadvantages and dangers For one thing large doses of the hormones for example 300 mg daily of cortisone may be necessary to control haemolysis let alone to bring about clinical cure Secondly the drugs may have to be used for many months or even years For this reason it is wise to use only the

minimum doses which are capable of allowing the patient to maintain his haemoglobin at 10-12 g per 100 ml and not to try to raise the haemoglobin to the normal level The results of successful steroid therapy are illustrated in Figs 5 and 6

Fig 7 illustrates the initial good result of steroid therapy in a patient whose acquired haemolytic anaemia was secondary to chronic lymphatic

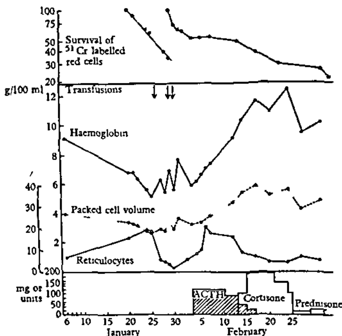


Fig 6 Effect of treating with ACTH cortisone and p edn sone a woman ag d 70 who had an id opathic acquired haemolyt c anaemia of the warm antibody type

leukaemia It illustrates how after initial great benefit the patient died quite suddenly from pneumonia an experience which underlines the well known risk of serious infections in patients being treated with high doses of steroids

Steroids have also been used in the treatment of patients suffering from the cold antibody type of haemolytic anaemia Some appear to benefit slightly as indeed they should if the hormones can suppress antibody formation However clinically it is difficult to assess their benefit for the patients tend to improve on bed rest alone no doubt owing to their being kept warm

In view of the risks of long continued therapy with high doses of steroids I feel that this should not be persisted in in patients with the cold antibody type of haemolytic anaemia The syndrome is usually a very chronic one and even if the patients do not get better spontaneously at least they often live for years without getting appreciably worse

I shall now deal briefly with the practical management of patients having acquired haemolytic anaemia of the warm antibody type, with particular

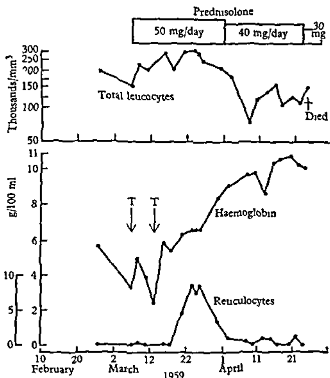


Fig 7 Effect of treating with prednisolone a man aged 65 suffering from chronic lymphatic leukaemia and auto immune acquired haemolytic anaemia of the warm antibody type *After a good response he died quickly of fulminating pneumonia T = transfusion*

reference to when if at all splenectomy should be carried out If the patient is seriously anaemic particularly if his haemoglobin is falling large doses of steroids should be given without delay—in an adult 300 mg of cortisone or 60 mg of prednisone daily Improvement should be obvious within five days If nothing seems to be happening ACTH 80 units daily may be given in addition Most patients respond to some extent some dramatically and depending on the degree of response the doses of steroids should be decreased with a view to maintaining the patient on a

low dose say 50 mg of cortisone daily and of stopping the dose altogether eventually If after 6–8 weeks of high dosage steroid therapy the patient is still moderately or seriously anaemic splenectomy should be considered The survival of his own red cells in his own circulation should then be estimated together with surface counting If the spleen is demonstrably an important site of haemolysis and the liver an unimportant site splen ectomy should be carried out with the expectation of major improvement and with the hope that the dosage of steroid could be reduced or the patient taken off the drug altogether

With regard to blood transfusions I think it is fair to say that they are of relatively little use in the treatment of acquired haemolytic anaemia except in the rare instances when the patient s auto antibody is a specific one and it is possible to transfuse the patient with red cells lacking the relevant antigen

In most instances transfusion in a patient who is failing to compensate for the haemolysis merely provides him with more red cells to destroy and any benefit is short lived A very serious situation arises if equilibrium cannot be achieved with massive steroid therapy and transfusions appear to be imperative in order to prevent the patient dying from anaemia It is in these patients that splenectomy will probably be undertaken as a last resort but as these are patients who are probably producing very large amounts of auto antibody or in whom the antibody is an auto agglutinin the chances of a successful outcome are poor

Finally I should say something on aetiology Throughout this paper I have been using the term auto antibody to describe the proteins adsorbed to the patient s red cells in cases of acquired haemolytic anaemia and I think nowadays few people would dispute the fact that this disease is one in which the normal processes of immunological tolerance have broken down with the result that antibodie are produced capable of affecting adversely normally occurring antigens—in particular antigens on the surface of the red cells How does this take place?

Animal experiments have as yet failed to provide the answer One possible mechanism has been recently enunciated by Campbell (1957) He located the primary fault in the red cell surface which he suggested might become abnormal as the result of faulty protein synthesis or of adsorption of antigenic fragments from some infective agent or denatured autologous protein He further postulated that the alteration to the red cell surface might lead to the formation of antibodies capable of cross reacting with normal red cells An alternative explanation is that the antibody forming tissues may as the result of a pathological process for example neoplasia lose their foetally acquired tolerance of red cell antigens This latter hypo

thesis is an attractive one and might well be the explanation for the development of acquired haemolytic anaemia in the course of chronic lymphatic leukaemia or reticulosarcoma which are neoplasia involving potential antibody forming cells

An extension of this hypothesis is one in which somatic mutation occurring in antibody forming cells might in the absence of neoplasia lead to the presence of 'forbidden clones' of antibody forming cells to use Burnet s (1959) phraseology, capable of forming continuously or for relatively long periods of time, anti red cell antibodies Personally I think it can hardly fail to be significant in this respect that in auto immune haemolytic anaemia the antibodies appear often to be non specific , which may be only another way of saying that they are imperfect in some way— many of them may in fact be imperfect Rh antibodies Such imperfection would I should have thought, be anticipated, if the antibodies were formed by aberrant or neoplastic antibody forming tissue it would be less likely if the antibodies were developed as an immune response to heterologous stimuli

The cause of the formation of the cold type of antibody is also obscure but here there are hints that the antibodies may not be auto antibodies but instead cross reacting antibodies of heterologous origin The antibodies for instance react strongly with rabbit red cells and characteristically they may be formed as a transient apparent immune response following virus pneumonia Nevertheless they also occur for no apparent reason as in the cold haemagglutinin syndrome and also in association with reticulo sarcoma In the cold haemagglutinin syndrome large quantities of anti bodies are usually continuously formed over a period of years and somatic mutation of antibody forming cells provides a plausible but unproved explanation for this But here, too it seems impossible to exclude the possibility that the original exciting agent for antibody formation was of heterologous origin

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IRON METABOLISM AND THE TREATMENT OF IRON-DEFICIENCY ANAEMIA

WILLIAM M DAVIDSON

INTRODUCTION

Although iron is the second most common mineral and is found almost universally paradoxically lack of iron is one of the commonest deficiencies suffered by man At first sight it is almost inconceivable that when even the poorest diets in this country contain roughly ten times the daily requirement the tissues can be starved of this essential material so frequently but this is one of the many riddles connected with iron metabolism

The late Sir Lionel Whitby in his superb Thomas Huxley lecture delivered at Charing Cross Hospital in 1954 quoted Huxley as having said My business is to teach my aspirations to conform themselves to fact not to make facts harmonise with aspirations Sit down before fact as a little child be prepared to give up every preconceived notion follow humbly to wherever and whatever abysses nature leads or you will learn nothing Huxley s precept has much to commend it to investigators of iron metabolism Although the subject has been studied more intensively than any *other in this field of medicine and many of the problems have been reduced* to mathematical exactitudes there still remains much to be learned, and perhaps to be unlearned before we can understand completely the mechanism of iron absorption, transport storage incorporation and function

IRON COMPOUNDS AND OXYGEN METABOLISM

We have only to consider the iron containing substances scattered throughout the animal and vegetable kingdoms to realize the importance of this metal to life In the ionic state iron would cause severe toxic effects and within the living tissues it must be conjugated with protein to control and *direct its activity Such conjugates form two groups the simple iron* transferring and storage group including in man transferrin ferritin and haemosiderin where the iron is isolated by being enveloped within the protein molecule and the more complex oxygen transferring or storing group with the iron chelated into a haem or haem like porphyrin ring structure lying readily accessible on the surface of the protein The latter

group can be further divided into those conjugates like haemoglobin and myoglobin which combine reversibly with oxygen without any valency change and those including cytochrome and the respiratory enzymes such as cytochrome oxidase peroxidase and catalase where the iron becomes alternately reduced and oxidized in the transferring process

This difference apparently depends upon the method of attachment of the iron to the protein If one of the links between the iron and imidazole rings of histidine residues in the globin is weak this will break when the surrounding oxygen concentration is high allowing oxygen to displace the imidazole ring to form oxyhaemoglobin or oxymyoglobin The new link however is also weak and the process is easily reversed when the partial pressure of oxygen falls In cytochrome and the respiratory enzymes all the linkages are strong so that no displacement of imidazole rings is possible but at the same time the iron is also more accessible and undergoes alternating valency changes during the transfer of oxygen (Martell & Calvin 1952)

In addition variations in the structure of the side chains projecting from the porphyrin rings affect the oxygen combining properties The prosthetic group of chlorocruorin the red green iron containing pigment found in the body fluid of certain marine worms only differs from that of haemoglobin by the oxidation of one of the vinyl side chains and yet it has a lower affinity for oxygen Interaction between the prosthetic groups and changes in the globin can also affect the relationship Haemoglobin with four haem groups has a different oxygen dissociation curve from myoglobin where the molecule resembles one quarter of the haemoglobin molecule with only one prosthetic group (Rossi Fanelli 1948 Schmidt 1959)

Differences in the physico chemical structure thus determine the reaction and particularly the affinity of the substance for oxygen No doubt each of these haem and haem like compounds has its own characteristics and is suited to the function it carries out The affinity of myoglobin is six times that of haemoglobin and that of the cell enzymes is even greater a mechanism well suited to provide the gradient necessary for the transfer of oxygen from the alveolar air to the ultimate site of cellular respiration in the sarcosomes (Schmidt 1959) Chlorocruorin is probably adapted to maintain respiration despite the low oxygen content of the sea bed slime where the sluggish worms using it live and may well have a functional relationship with cytochrome a which has similar prosthetic groups (Vannotti 1959)

In human beings there are a number of these compounds but they are not specific and are found in other species and even other organisms Haemoglobin is the pigment in the blood cells of all vertebrates but it is also found in the body fluid of lowly worms in insects and even in the roots

of leguminous plants (Keilin & Wang 1945) This is perhaps understandable on functional grounds but many anomalies such as the mixture of haemoglobin and chlorocruorin found in the body fluid of the marine worm *Serpula* (Fox 1949) remain unexplained

It is not clear why iron has been used so widely as the metal for oxygen transfer Other heavy metals can function in the same way (Martell & Calvin 1952) and copper is present in the blood of some crustaceans and the ink fishes as haemocyanin a blue coloured protein porphyrin like complex endowed with the property of absorbing oxygen reversibly No doubt under the conditions of life in the vertebrates haemoglobin is more efficient It is of interest that in man the concentrations of iron zinc, and copper in the plasma are approximately the same (Spector, 1956) that all three are transported bound to protein that the levels of iron and copper are inversely related and that these metals form the essential links in many enzymes for example copper in tyrosinase and zinc in carbonic anhydrase

IRON IN MAN

Iron is found in man in four forms as a ferric protein conjugate in the storage iron particularly in the cells of the intestinal mucosa and the reticuloendothelial system and as iron in transit bound to the β_1 globulin of the plasma in the complex haem prosthetic group in the molecules of the haemoglobin of the red cells and their immediate marrow precursors and in the myoglobin of the muscles in the haem like groups in the molecules of cytochromes and enzymes of the blood and tissue cells and as ferrous iron in the process of being transferred across cell membranes from one ferric complex to another

By means of the electron microscope the storage iron can be seen to consist of tetrads formed by the polymerization of iron protein conjugates In the soluble ferritin these tetrads are spread freely through the cell and give a diffuse blue staining with the Prussian blue reaction In the reserve stores of the less soluble haemosiderin the tetrads are aggregated into visible granules To transfer iron from one protein conjugate to another particularly across cell membranes it is necessary to convert it to the ferrous form by enzymatic action Thus xanthine oxidase is thought to be the enzyme which shifts iron from the apoferritin in the cells of the intestinal mucosa to the iron binding protein of the plasma (Green & Mazur 1956) and ferrochelatase the enzyme which transfers iron to the porphyrin ring as the final stage of haemoglobin synthesis (Rimington 1959)

IRON METABOLISM

Iron is a toxic substance and the intravenous injection of 10 mg or more of ferrous iron in an ionizable form is liable to cause symptoms. That is the quantity which will just exceed the power of the plasma to bind it into a protein complex. For this reason the body has to be protected against abnormal quantities of iron and the whole economy is based upon conserving a definite quantity of iron and using and re-using this quota. Only

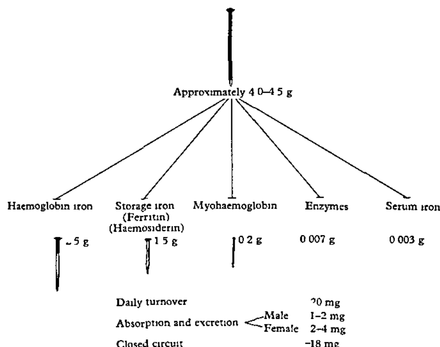


Fig. 1. A diagram to show the distribution of iron in the body. The quantities are illustrated by nails of the correct weight × ½

just enough iron is absorbed to meet losses whether accidental or physiological. The total amount of iron normally held in the body lies between 4 and 5 g. This is partitioned into 2·5–3·0 g in the haemoglobin, 1–1·5 g in the reserve stores as haemosiderin and ferritin, 0·2 g as myoglobin, 0·007 g in the iron containing enzymes and 0·003 g in the serum iron (Fig. 1).

The average daily turnover of some 25 mg means a change in serum iron every 8 hr. The iron for this is mainly provided by the breakdown of the 0·8% of the red cells which reach the end of their life span each day. During the breakdown the haem rings are split open and the iron liberated

of leguminous plants (Keilin & Wang 1945) This is perhaps understandable on functional grounds but many anomalies such as the mixture of haemoglobin and chlorocruorin found in the body fluid of the marine worm *Serpula* (Fox, 1949) remain unexplained

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From the plasma most of the iron is transferred to the reticulo endothelial cells especially in the bone marrow where it enters the labile iron pool ready to be used in haemoglobin synthesis A small amount is exchanged with the permanent reserves the haemosiderin and a trifle with the tissue iron mainly for the synthesis of myoglobin and enzymes

According to Bessis & Breton Gorius (1959) the next stage in iron metabolism involves direct contact between the reticulo endothelial cells and the developing erythroblasts for the transfer of iron Their theory is that the iron is transferred by the donation of particles to the erythroblasts that is by pinocytosis or as they call it ropheocytosis The phenomenon of grouping of the erythroblasts round the reticulo endothelial cells has been known for long (Plate 1) but its significance has been interpreted differently In a marrow with active erythropoiesis every group of erythroblasts has one or more macrophages in its midst and certainly the erythroblasts are not limited to late normoblasts as might be expected if this were a phenomenon somewhat comparable to a cloakroom and the cells were merely handing in their unwanted nuclei This new theory however requires to be reviewed in accordance with Huxley s precept for there are many difficulties for example it does not explain the transfer of iron to haemoglobin *in vitro* in blood containing many reticulocytes but presumably without iron laden macrophages (Goldberg 1959)

Whether the iron diffuses into the erythroblasts or is delivered there in bulk it still requires to be reduced to the ferrous form before it can be incorporated into the haem Suitable reducing agents have been shown to be dehydroascorbic acid glutathione and ergothionine and these are present in the corpuscles in quantities comparable to the optimal concentrations found *in vitro* (Goldberg 1959)

There is some argument as to whether globin and haem are formed synchronously or in series (Rimington 1959) but for our present purpose the main interest is in the prosthetic groups In the erythroblasts haem is synthesized from succinate and glycine through the stages of α amino β keto adipic acid β amino levulic acid porphobilinogen and protoporphyrin Ferrous iron is required at two stages in this process for the formation of α amino β keto adipic acid when iron containing enzymes are involved and again in the final stage where the iron itself is chelated into the protoporphyrin rather as a jeweller sets a diamond into the claws of a ring A special enzyme descriptively named ferrochelatase transfers the iron to the porphyrin (Rimington 1959)

For rapid oxygen exchanges the iron in haemoglobin must be maintained in the ferrous state and any appreciable oxidation to the inactive form methaemoglobin must be prevented by a reducing system It has been

Almost the whole of this iron goes directly to the labile pool in the reticulo endothelial cells of the bone marrow and is later transferred to the erythroblasts to form new haemoglobin Only a small portion returns from the labile pool to the circulating plasma The amount of iron used in the tissue enzymes is small, and the replacement of the myoglobin is so slow that neither of these contributes more than a negligible amount to the iron turnover

In man the loss of iron from the body is almost solely as iron in cells and cellular debris desquamated from the skin, urinary and alimentary tracts The total loss by this means is under 1 mg daily, but in women during the reproductive years this is doubled by the loss of some 30–70 ml of blood containing on the average some 25 mg of iron with each menstrual period

In normal adults the iron lost is exactly balanced by absorption from the alimentary tract but in children there has to be a positive increment to allow for the requirements of growth and in pregnancy a similar gain is necessary to provide for the needs of the foetus Part of the iron in the food is liberated by digestion and as simple ferrous salts or possibly as ferrous amino acid complexes this is absorbed into the epithelium of the duodenum and upper jejunum and to a decreasing extent lower down towards the ileum Another part of the iron links with phosphates or phytic acid to form insoluble compounds which cannot be absorbed The exact relationship between the gastric juice and iron absorption is difficult to understand It was thought that achlorhydria led to iron deficiency anaemia and certainly extensive gastric resections are frequently if not always, followed by hypochromic anaemia Using newer techniques including biopsy of the gastric mucosa and iron absorption studies it seems that the achlorhydria may be fortuitous or actually the result of the iron deficiency (Davidson & Markson 1955 Moore & Dubach 1956)

Once within the mucosal cells the bivalent ferrous iron is oxidized to the trivalent ferric form and enclosed in a mass of protein the apoferritin to form the ferritin In this inactive form the iron remains in the mucosal cells for a time and is then transferred to the plasma This transfer necessitates reduction of the iron possibly by the enzyme xanthine oxidase (Green & Mazur 1956) and re oxidation to link it to the β_1 globulin to form transferrin (Siderophilin) Normally the plasma contains between 80 and 160 μg of iron per 100 ml bound in this way the serum iron but the globulin is less than half saturated and can carry up to between 300 and 350 μg of iron This latter figure is the total iron combining power of the plasma and the difference between the two is the unsaturated iron com bining power

PLATE I



Group ng of the normoblasts round a macroph ge in a bone m rrow smear Jenner–Giemsa × ~oo

suggested that within the red cells this is obtained by the interaction of a riboflavin protein complex, diaphorase 1 and coenzyme 1 (Gibson, 1948) or alternatively an enzyme system involving glutathione has been postulated (Eggleton & Fegler, 1952)

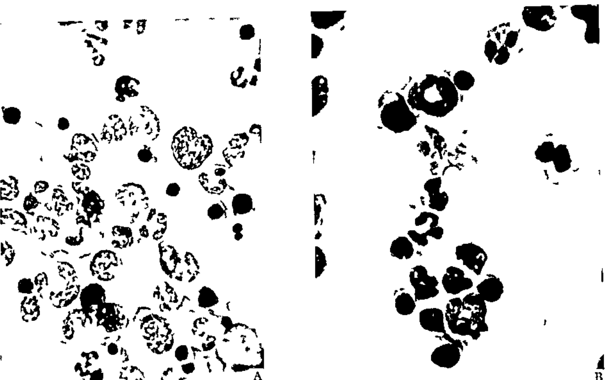
Incorporation of iron ceases with the maturation of the reticulocytes to erythrocytes and it is believed that the haemoglobin iron then remains incarcerated within the red cells throughout their life span a fixed concept which, in view of iron studies in animals, may require revision in terms of Huxley s dictum (Gower 1959) Another difficulty is that although the average life span of the red cells is 110–120 days, a proportion of the haemoglobin iron reappears in the circulation prematurely Probably some of the normoblasts are destroyed prematurely These may be the cells seen grouped round the macrophages by Bessis (1959) for the macrophages which appear in droves whenever there is increased marrow activity can then be seen to be phagocytosing and destroying a proportion of the normoblasts (Plate 1)

Towards the end of their life span when their enzyme systems have deteriorated and can no longer provide the energy necessary to maintain their integrity the red cells are engulfed by the macrophages of the reticulo-endothelial system particularly in the spleen Enzymatic digestion probably lasting only a few minutes frees the haemoglobin and splits open the porphyrin ring to verdohaemoglobin With the subsequent removal of the iron the chain of pyrrol rings becomes bilirubin and is excreted through the liver The iron oxidized to the ferric form is bound to protein as transferrin and rejoins the metabolic circle to be carried to the marrow and incorporated into the haemoglobin of fresh cells a reincarnation reminiscent of the method of choosing a successor to the Dalai Lama In its sojourn only traces of the iron, contaminating the bile or locked in desquamated cells have been lost

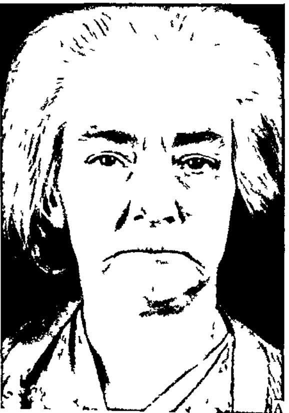
A similar haemoglobin breakdown occurs in the cells destroyed prematurely and this accounts for some of the bilirubin discarded at the beginning of studies in which the haem has been labelled with ^{14}C or ^{15}N glycine (London, West, Shemin & Rittenberg 1950)

METHODS OF INVESTIGATION

Iron metabolism is often investigated by an indirect method where the amount of iron available is taken to run parallel with changes in the quantity of haemoglobin in the blood This is not really applicable to normal individuals unless combined with deliberate phlebotomy but has been used extensively in assaying different methods of therapy The most direct

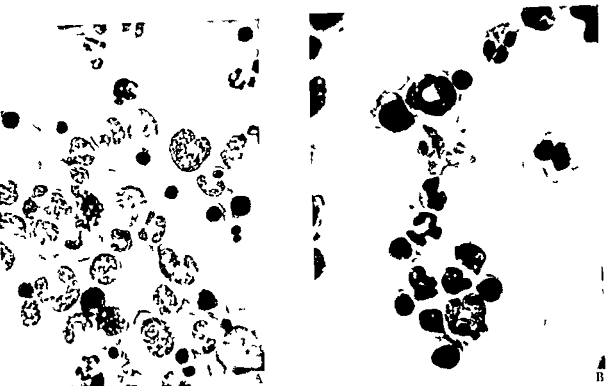


The m cro normobla ts and the g ant metamyelocytes in l one marrow mears A from the case llu trated in I l te 2 B from a imilar ca e of iron leficiency Jenner Giem a ×700





A, the facies to show the dry brittle hair, the tired eyes and the cracks at the angle of the mouth; and B, the smooth sore tongue in iron deficiency



The micro normoblasts and the giant metamyelocytes in bone marrow smears. A, from the case illustrated in Plate 2; B, from a similar case of iron deficiency. Jenner-Giemsa. ×700

method against which all other methods must be calibrated is the iron balance This is a tedious investigation requiring the estimation of the whole of the iron intake and excretion over a period of time It can be supplemented by such measurements as estimation of the serum iron the unsaturated iron combining power of the serum the total body haemo globin mass derived from the blood volume and haematocrit estimations and the available stores of iron estimated from marrow sections (Davidson & Jennison 1952)

Although providing the basic facts there are obvious limitations to these methods and much information especially regarding the dynamic aspects of the problem has been derived from isotope labelling methods Red cells labelled *in vitro* with ^{51}Cr can be reinjected and used to determine the red cell mass and indirectly in conjunction with the haematocrit estimation the blood volume or to follow the life span of the cells More information can be obtained with the iron isotopes ^{59}Fe which has a half life of 46 days and is commonly employed or ^{55}Fe with a half life of 2 9 years The ^{59}Fe in tracer doses can be used to label iron salts to be given by mouth or it can be introduced into foodstuffs such as eggs meat or vegetables by administering it to the animals or incorporating it into the medium upon which vegetables are grown (Moore & Dubach 1956) It may also be given intravenously The labelled iron administered in one of these ways is built into the haem of new cells and can be demonstrated in the peripheral blood after a few days The degree of incorporation of the isotope into the red cell haemoglobin can be calculated and by estimating the portion of an orally administered dose lost in the stools the percentage absorption is obtained The labelled red cells can also be followed to determine such features as their life span ^{55}Fe is used only as a second tracer In the technique of double labelling advantage is taken of the fact that these two isotopes are easily separated When the ^{55}Fe is given intravenously and the ^{59}Fe orally the ^{55}Fe indicates the percentage incorporation of iron into the red cells Assuming that ^{59}Fe is incorporated to the same degree the percentage absorption can be calculated This method has provided particularly important information regarding absorp tion for it has eliminated the difficulty encountered in the previous method through abnormal sequestration of iron (Bothwell Pirzio Biroli & Finch 1958) The β_1 globulin of the plasma may be made to take up the isotope *in vitro* and this labelled material used to estimate the plasma volume or in iron metabolism studies

For comparative purposes the porphyrin ring of the haem instead of its iron may be marked by using glycine marked with ^{15}N (Muir Neuberger & Perrone 1952) or ^{14}C These methods require chemical separation of

the porphyrin, or its derivatives before the isotope concentration can be determined the former, which is not radioactive, by the mass spectroscope and the latter, as with the ^{59}Fe, by a scintillation counter The globin is also labelled and in certain investigations it is an advantage to separate it off and estimate its activity

Radioactive isotopes particularly ^{59}Fe can also be used in conjunction with surface counting By means of counters placed over the heart liver, spleen and sacrum the distribution of the labelled iron can be followed Soon after absorption, when the iron is circulating freely in the plasma, the counts are the same over all the areas but within an hour or two the activity of ^{59}Fe becomes concentrated in the bone marrow in accordance with what would be expected This phase is followed by the entry of labelled cells into the circulation and in a few days the distribution of the iron becomes more general again as the labelled blood circulates Later and particularly when there is haemolysis phagocytosis of the red cells increases the activity over the spleen

THE CONTROL OF IRON ABSORPTION AND IRON METABOLISM

The controlling factors have not yet been completely elucidated, but certain facts are well established In man the daily absorption of iron from a diet containing about 15 mg is 1–2 mg which just balances the daily loss including menstruation In anaemia particularly iron deficiency anaemia the absorption is much more efficient and may rise to 30% of the iron intake

For a time it was firmly believed that the absorption of iron was regulated by the process of mucosal block (Granick, 1951) The idea was that the accumulation of ferritin in the mucosal cells prevented any further uptake Heilmeyer and his co workers (Heilmeyer Keiderling & Wohler 1957) have obtained direct experimental proof that a high ferritin content in the duodenal mucosa does not in itself completely block further iron absorption although it is true that with increased iron dosage the proportion of iron absorbed is diminished The mucosal block theory also fails to explain why iron continues to be absorbed in untreated pernicious anaemia haemolytic anaemias and even in conditions of iron overloading including haemochromatosis and experimental pyridoxine deficiency in pigs (Moore & Dubach 1959) Some such mechanism may delay absorption but it does not prevent it completely

The degree of anaemia the oxygen tension and the plasma iron levels have all been eliminated as factors regulating absorption (Moore & Dubach 1959)

The amount of iron available in a *soluble ferrous state in the duodenum* is important and absorption is linked directly with the absorption of traces of copper and inversely to the intake of pyridoxine It seems important too that absorption is increased when erythropoiesis is stimulated but no direct linkage with the marrow stimulating factor erythropoietin has been established Absorption is usually inhibited when adequate iron stores are present in the tissues but this is not invariable and the mechanism is certainly not clear

Drabkin (1951) has emphasized the importance of a linkage between the hormones particularly thyroxine and the cytochrome c in the tissue cells and regards this as the mechanism whereby the hypothalamic region of the brain ultimately controls oxygen consumption by the tissues Such a mechanism could have a profound influence upon iron metabolism and might regulate the serum iron level through the ferritin storage mechanism of the reticulo endothelial cells (Schaefer 1959)

Proteins play an important part in the control of the next phase of iron metabolism (*Laurell 1959) The iron binding β_1 globulin of the plasma* is particularly important In haemolytic states a new mechanism appears and the haptoglobins of the plasma bind free haemoglobin and prevent its loss through the kidneys or when the potentialities of this method are exceeded the haemoglobin is oxidized to methaemoglobin and linked with albumin to form methaemalbumin In the tissues too the protein apo ferritin always appears where it is required to bind iron These controlling proteins may be pathologically altered and one effect seen in toxic states *is to bind too little iron in the plasma and too much in the tissues Although* the liver plays the major part in the formation of the albumin fractions and the lymphoid tissues in the globulins their formation does seem to be linked and it is of interest that Schedl & Bartter (1959) discovered accidentally that the infusion of human serum albumin depressed the iron binding capacity of the serum

The regulating mechanism appears to break down at an even later stage in refractory sideroblastic anaemia where Bessis & Breton Gorius (1959) have demonstrated the accumulation *of iron within the mitochondria of the* erythroblasts

DISORDERS OF IRON METABOLISM

The commonest disorder of iron metabolism is that caused by loss of iron from the body through haemorrhage The effect of a single haemorrhage even if severe in a normal individual with ample iron stores is transitory (Fig 2) As soon as compensatory blood dilution has been achieved the marrow is stimulated to increased erythropoiesis and the ferritin stores and

haemosiderin reserves are mobilized to meet the demand The plasma iron exchange is accelerated, in a few days the stimulation of erythropoietic activity and increased haemoglobin formation reach a peak and many young cells are poured into the circulation In a few weeks the haemoglobin level reaches the normal range and the erythropoietic activity declines Eventually recovery is complete except for the stores of iron which are only replaced very slowly over months or years

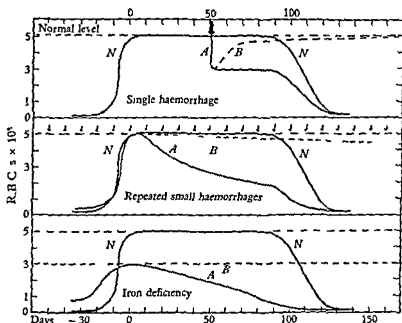
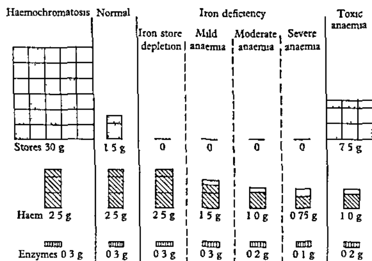


Fig 2 Diagrams illustrating the development of iron deficiency anaemia For comparison the normal life span of a Cohort of labelled cells *N* is shown in each diagram *Above* The effect of a single severe haemorrhage indicated by the arrow on a population of labelled cells *A* The dotted line *B* shows the recovery due to the formation of increased numbers of new cells which modifies the changes in the total red cell population *Middle* Repeated small haemorrhages indicated by the arrows reduce the labelled population As soon as the *iron stores have been exhausted the total red cell population dotted line B* also begins to diminish as iron deficiency anaemia develops *Foot* Once iron deficiency is established although the marrow activity is increased delay in the maturation of the red cells interferes with production The cells in the labelled population *A* show the effects of continued blood losses and a slightly shortened life span At the same time the total red cell population dotted line *B* is now reduced

Repeated blood losses such as those due to chronic bleeding from the gastro intestinal and urogenital tracts soon lead to exhaustion of the iron stores (Fig 3) Thereafter in the absence of treatment a precarious balance may be established (Fig 2) *The available iron is more fully absorbed than* normally the transport of iron is accelerated and the total haemoglobin formation may be normal or even increased Despite this the amount of haemoglobin formed is insufficient to meet the increased demand and in

the hyperplastic marrow the individual erythroblasts develop to poor ragged half haemoglobin starved micronormoblasts which give rise to hypochromic microcytic red cells mere shadows of the normal Many of the defective red cells are never born into the circulation but are destroyed in the marrow and even those which achieve their objective have a shortened life span (75–90 days (Pollycove 1959)) Eventually as the condition progresses the marrow becomes hypoplastic



F g 3 The iron distribution in the body in the normal state n haemochromatosis during the development of iron deficiency anaem a and in the anaem a of toxic infective cond tions In iro i deficiency the iron stores re exhausted first then usually the haemo globin is reduced progressively and fin lly the iron cont ining enzymes suffer The massive accumulation of iron in haemo hromatosis is asso i ted with normal h emoglobin forma tion but n the toxic inf ctive states haemoglobin formation and possibly the enzymes are di turbed Each squ re represents 1 g of iron

Without treatment such patients can exist for a long time with a very severe degree of anaemia Gradually the other iron containing complexes are affected the iron enzymes (Goldberg 1959) and the myoglobin and possibly secondarily also other enzyme systems (Beutler 1959) This adds to the sluggishness weakness and stubborn mentality which these patients often exhibit All cells but especially those which have a short life span and have to be replaced frequently suffer The hair greys becomes dry and brittle and may fall out even in young women the nails break easily and tend to be spoon shaped possibly through secondary interference with cystine metabolism (Jalili & Al Kassab 1959) the mucous membranes become thin and the tongue glazed and sore the edges of the mouth crack (Plate 2) and atrophy of the oesophageal surface causes

haemosiderin reserves are mobilized to meet the demand The plasma iron exchange is accelerated, in a few days the stimulation of erythropoietic activity and increased haemoglobin formation reach a peak and many young cells are poured into the circulation In a few weeks the haemoglobin level reaches the normal range and the erythropoietic activity declines Eventually recovery is complete, except for the stores of iron which are only replaced very slowly over months or years

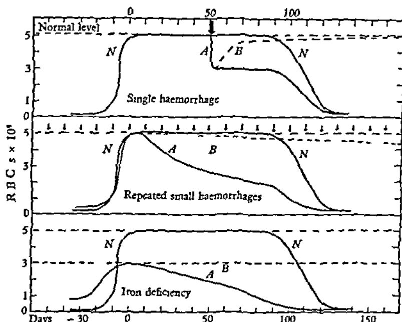


Fig 2 Diagrams illustrating the development of iron-deficiency anaemia For comparison the normal life span of a Cohort of labelled cells N is shown in each diagram *Above* The effect of a single severe haemorrhage indicated by the arrow on a population of labelled cells A The dotted line B shows the recovery due to the formation of increased numbers of new cells which modifies the changes in the total red cell population *Middle* Repeated small haemorrhages indicated by the arrows reduce the labelled population As soon as the iron stores have been exhausted the total red cell population dotted line B also begins to diminish as iron deficiency anaemia develops *Foot* Once iron deficiency is established although the marrow activity is increased delay in the maturation of the red cells interferes with production The cells in the labelled population A show the effects of continued blood losses and a slightly shortened life span At the same time the total red cell population dotted line B is now reduced

Repeated blood losses such as those due to chronic bleeding from the gastro intestinal and urogenital tracts soon lead to exhaustion of the iron stores (Fig 3) Thereafter in the absence of treatment a precarious balance may be established (Fig 2) The available iron is more fully absorbed than normally the transport of iron is accelerated and the total haemoglobin formation may be normal or even increased Despite this the amount of haemoglobin formed is insufficient to meet the increased demand and in

including rheumatoid arthritis Characteristically there is a low serum iron level but in contrast to simple iron deficiency there is also a low serum iron combining power (Fig 5) Despite this or as a result of this there is a rapid turnover of serum iron and stocks accumulate in the reticulo endothelial cells (Fig 3)

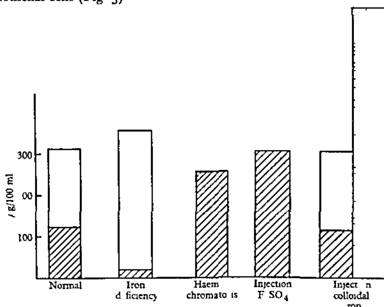


Fig 4 The iron carrying power of the plasma in the normal state and in disorde s of iron metaboli m together with the effect of ionic iron (FeSO) and bound iron administered intravenously The bound iron is carried by its own binding m terial and does not at first materially alter the plasma White = unsaturat d iron-combining power hatched = s rum iron

The next stage in iron metabolism the transference of the iron from the reticulo endothelial cells to the erythroblasts and its incorporation into the haemoglobin may also be affected As a result iron accumulates in the reticulo endothelial cells and even in the erythroblasts Such a condition may be encountered in the reticuloses including Hodgkin s disease but it is most characteristic of the congenital hypochromic anaemias of the thalassaemia type Thalassaemia itself would appear to be due to an enzyme defect causing an error at a very late stage of haem formation perhaps even the final incorporation into the molecule by the chelatase (Bannerman Grinstein & Moore 1959)

In the case described by Garby (Garby Sjolin & Vahlquist 1957) the defect in haem synthesis seemed to have been at an earlier stage for the lack of iron utilization was accompanied by an accumulation of the earlier precursor coproporphyrin in the red cells

the difficulty in swallowing of the Patterson-Brown-Kelly syndrome Secondary inflammatory invasion of the oesophageal wall leads to scarring and web formation, while in a proportion of cases a post cricoid carcinoma may eventually develop The gastro intestinal mucosa is severely affected, achlorhydria is established, and ultimately atrophy of the gastric mucosa, from which no recovery is possible supervenes It is possible that the tissue changes precede the development of the anaemia more frequently than is suspected

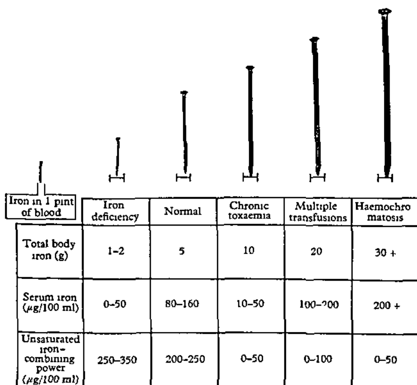
Leucopoiesis is also affected, and after an initial stage of overactivity, it shares in the marrow failure and a leucocytosis is followed by a neutrophil shift to the right Giant metamyelocyte formation in the marrow may indicate a secondary deficiency, but it is more probably a direct effect of iron depletion (Plate 3)

Once fully established the changes in the gastro intestinal tract inhibit spontaneous recovery The part played by achlorhydria in iron deficiency is difficult to evaluate It has been fully investigated by Davidson & Markson (1955) Badenoch, Evans & Richards (1957) and Moore & Dubach (1956) and while achlorhydria may play a part in preventing the optimum conversion of the iron available in the diet to the ferrous form and so diminish absorption it does not prevent the absorption of iron, either from foodstuffs or from therapeutically administered salts The effect may be to reduce the amount of iron absorbed from a deficient diet to below the required level or more probably to prevent increased absorption when the demand for iron is raised Operative removal of a large part of the stomach has a more definite effect and may produce an iron deficiency anaemia occasionally completely refractory to the ordinary oral therapeutic measures, but responding to parenteral iron (Hobbs & Discombe, 1959) The dimin ished absorption may well be due to the loss of the fundus of the stomach which acts as a hopper for holding the iron containing food until it is ready to be absorbed (Witts 1958) or alternatively the alteration in the alimentary canal may result in peristaltic hurry and insufficient time for absorption

Loss of absorption also occurs in disorders of the small intestine such as have been grouped as non tropical sprue and coeliac disease The latter is of course particularly interesting in its relationship to sensitivity to gluten or more strictly to its gliadin fraction (Sheldon 1958) Here failure to absorb iron is probably just part of a general failure to absorb but the *possibility of a specific factor cannot be excluded*

Errors of protein formation particularly if they affect the plasma proteins interfere with iron metabolism and in conjunction with changes in the reticulo endothelial system provide the basis for a type of anaemia often found in the infective and toxic states and the collagen' diseases

experimental animals doubt has been cast upon the part played by iron in the pathogenesis of the cirrhosis of the liver and pancreas Although vast quantities of iron accumulate in the tissues of patients with refractory anaemias who have been given over thirty pints of blood or a great excess of parenteral iron fibrosis is uncommon and the condition seldom progresses to bronzed diabetes When there is fibrosis a careful check on the



Iron in 1 pint of blood	Iron deficiency	Normal	Chronic toxaemia	Multiple transfusions	Haemochromatosis
Total body iron (g)	1–2	5	10	20	30 +
Serum iron (μg/100 ml)	0–50	80–160	10–50	100–200	200 +
Unsaturated iron-combining power (μg/100 ml)	250–350	200–250	0–50	0–100	0–50

Fig 5 The total body iron in the normal and disordered states illustrated by nails of the appropriate weight × ½

quantities has usually shown more iron in the tissues than could have reached them from the transfused blood Similarly despite many attempts no liver cirrhosis has been produced by the administration of iron to experimental animals (Oliver 1959) As a result Cappell (Cappell Hutchison & Jowett 1957) felt that there must be some fibrosing factor associated with the iron absorbed from the bowel possibly traces of copper which causes the cirrhosis of the liver and interferes with the islets of Langerhans and the other endocrine organs The difference between transfusional siderosis and

Iron metabolism in hypoplastic and especially aplastic anaemia reflects the almost complete lack of haemoglobin synthesis with serum iron exchange nearly at a standstill With the absence of the stimulus of active erythro poiesis iron absorption is limited and any accumulation of iron stores is very slow

In contrast, in haemolytic anaemia the iron turnover is very rapid particularly in the acquired varieties (Pollycove, 1959) where there is a great increase in 'ineffective haemoglobin formation , that is haemoglobin which never reaches the circulating red cells This is broken down almost at once and increases the rapidity of the iron turnover very greatly

In pernicious anaemia the greatly increased circulation of iron is again complicated by the extreme degree of '*ineffective haemoglobin formation* and as a *result the plasma iron level is usually high* despite the rapid removal of iron from the circulation In a severe case the effective incorpora tion of iron into the cells is relatively small but on treatment there is a rapid adjustment and even a tendency to swing towards the iron deficiency pattern

In the earlier stages of polycythaemia vera there is an increased iron turnover but of a normal pattern Later however this tends to be replaced by a haemolytic type of iron exchange

In contrast to iron deficiency there are the states in which enormous quantities of iron accumulate in the tissues haemochromatosis trans fusional siderosis nutritional siderosis and pulmonary siderosis Haema chromatosis seems to be an inherited defect of iron metabolism Although a number of cases have been described in sibs the evidence that there has been passage from one generation to another is not convincing Neverthe less it seems possible that there is a dominant hereditary predisposing factor and an acquired precipitating factor (Shapira & Dreyfus 1959) Increased absorption of iron over many years results in the accumulation of vast quantities of iron usually well over 30 g in the tissues (Figs 3 and 5) Increased absorption has not always been demonstrated in the fully developed disorder but it must have been present at one time to account for the enormous quantities of iron in store It seems probable however that the increased absorption is secondary *to a change in the tissues* especially the liver pancreas and endocrine organs which greatly enhances their avidity for iron (Gillman & Hathorn 1958) As would be expected with the vast stores of iron available the plasma iron carrying protein is usually saturated Despite these changes in iron metabolism haemoglobin formation tends to be unaffected so that in contrast to the general rule iron absorption is increased despite a normal marrow and large iron stores

As a result of the findings in transfusional siderosis and in iron loaded

cells typical of haemolytic anaemias are also found in the secondary haemolysis of chronic leukaemia and other disorders such as polycythaemia and myelosclerosis

TOXIC EFFECTS

Excessive quantities of iron in the tissue cause damage both by mechanical disturbance and by interfering with tissue respiration Ingestion of a large quantity of iron particularly by children not only damages the gastro intestinal mucosa but produces fantastic serum iron levels 3000 μg or more per 100 ml and results in profound shock leading to death in a few hours from extensive liver necrosis

The introduction of ionizable iron intravenously in quantities which exceed the iron binding capacity of the plasma causes acute toxic symptoms including an encephalopathy The modern intravenous or intramuscular iron preparations carry their own iron binding substance (Fig 4) and in such form vast quantities of iron up to 5000 μg per 100 ml can circulate without causing damage Nevertheless grossly excessive stores can be accumulated in this way and may cause tissue damage Large doses of saccharated iron lead to considerable deposition of ferritin in the tissues of experimental mice (Nissim 1955) and cause testicular atrophy Even more disturbing is the demonstration by Richmond (1959) that large doses of intramuscular iron initiate sarcomatous change in rats and mice Rats however are prone to such changes which have not so far been shown to occur in man Nevertheless the finding should be borne in mind in tempering enthusiasm for this type of therapy

TREATMENT

The treatment of iron deficiency anaemia has to be based upon our knowledge of the metabolism and the total dose of iron to be given more nearly approaches mathematical accuracy than is possible with any other form of therapy

The first principle in treatment is accurate diagnosis and this must include a search for continuing haemorrhage or signs of malabsorption from bowel disease These factors modify the approach to rational therapy and at an early stage they should be corrected as far as is possible

After a careful physical examination and possibly a quick capillary haemoglobin estimation to establish that there is an abnormality venous blood should be taken for the haemoglobin and mean cell haemoglobin concentration estimations and a blood film examined If there is anaemia that is a haemoglobin level below 13 5 g per 100 ml of blood (90%) in males or 12 g (80%) in females and there is no evidence of obvious bleeding occult blood should be looked for in the stools Thereafter if the

haemochromatosis may be due entirely to the length of time over which the iron has accumulated, in the latter probably forty to fifty years (Oliver 1959)

Disturbances of the mitochondria, with the accumulation of iron have been demonstrated by Richter (1957) using the electron microscope, and he suggested that the resulting enzyme disorders could lead to cell destruction Such a pathogenesis would bring haemochromatosis into close relationship with nutritional siderosis found especially in Africans In the former condition the error would be congenital possibly an enzyme defect while in the latter a similar acquired defect would result from their unbalanced, iron enriched maize diet (Gillman & Hathorn, 1958) This closely resembles the iron enhanced diet used by Goldberg & Smith (1958) to produce a heavy iron deposit in the tissues and what was interpreted as a tocopherol deficiency, in rats Tocopherol deficiency, in conjunction with a diet deficient in thioamino acids has been shown to lead to hepatic necrosis (Lindan & Himsworth 1950) and fibrosis could follow Thus these *conditions might depend upon one single defect, but so far the experi* mental proof is lacking

Paradoxically accumulation of iron in the lungs in the curious condition of pulmonary siderosis, is associated with an iron deficiency type of anaemia and the plasma changes typical of such an anaemia, a low serum iron and an increased unsaturated iron combining power Steiner (1959) has suggested that here the deposition of iron follows an antibody-antigen reaction in the lungs with liberation and accumulation of iron as a result of the haemolysis In addition to the iron lost by haemoptysis and the expectoration of iron laden macrophages the iron deposited in the lungs is not readily available for re utilization and an iron deficiency develops Radiological studies show that eventually much of the pulmonary iron is reabsorbed and presumably the iron deficiency would correct itself if there was no further haemolytic episode Whenever a case is available a careful *assessment of iron metabolism should be carried out* if Steiner is correct splenectomy should help but so far the results have not been too encouraging

Inhaled iron oxide dust although it causes dense shadows in the lung fields does not appear to be dangerous and as in pulmonary siderosis the shadows eventually clear (Perry 1955)

Except in the cirrhosis of haemochromatosis chronic liver disorders are usually associated with low serum iron levels (Napolitano & Scuro 1957) in contrast to acute hepatitis where the level is very high (Vahlquist 1959) and ferritin may be demonstrated in the plasma (Wohler 1959)

The accelerated exchange of iron and the splenic sequestration of red

Although 20–30% of the iron given is absorbed in the early stages of treatment of a severe iron deficiency this soon tails off to about 2% after the haemoglobin has reached normal levels This fact has to be borne in mind in calculating the relative cost of a course of treatment with parenteral iron A patient with an initial haemoglobin level of 7 5 g per 100 ml of blood lacks approximately 1 25 g of iron in the haemoglobin and possibly another 1 0 g for storage If the average rate of absorption over the first 8 weeks was 20% of the 100 mg given daily 1 12 g of iron would be available to replace the missing haemoglobin This is approximately the time needed for recovery (Swan & Jowett 1959) The subsequent accumulation of a store of 1 g at an average absorption rate of 2–3% would take over 50 weeks a figure which compares with the experience that it takes about a full year on oral iron treatment to reform the stores (Davidson 1957) The total cost of such a 50 week course of oral iron would vary from 2*s* 8*d* for ferrous sulphate to 30*s* for ferrous succinate The more expensive iron chelate tablets would be twice this price and an elixir might cost £12

With a haemoglobin level below 4 5 g (30%) the patient should have bed rest between 4 5 and 6 g (30 and 40%) much curtailed exercise but above this the restriction should be proportionately less as the haemoglobin level rises

With effective treatment iron deficient individuals should reach a maximum reticulocyte level which is too indefinite to be called a crisis about the 8th day After a short initial lag the rise in haemoglobin should be rapid the speed being related to the initial haemoglobin level Swan & Jowett (1959) have constructed curves giving the anticipated rise as a percentage of the original level These show that there should be a 50% reduction in the haemoglobin deficit in 18 days and that normal if not quite maximum levels should be reached in 8 weeks It is known however that the blood picture does not return completely to normal until many weeks have elapsed presumably until the end of the life span of the abnormal cells produced in the initial phase of recovery

If after 3 weeks treatment there has been no adequate response the situation must be reviewed under the following headings Has the patient been taking the iron regularly? Is there continued bleeding? If these can be eliminated Is there a defect in absorption? A defect in utilization? Some complicating factor such as uraemia or hypothyroidism? Is the diagnosis wrong?

The decision as to how long to continue treatment in iron deficiency could be established in theory by elaborate absorption and utilization studies but in practice it is better to have a rule of thumb method with the safeguard of repeated haemoglobin checks From the point of view of treatment

haemoglobin is over 9 g (60%) the M C H C 30% or under, and a film is in keeping with the diagnosis of a hypochromic microcytic anaemia treatment should be started at once with oral iron With a haemoglobin level of between 7 5 and 9 g (50–60%) a little more thought should be given to the diagnosis and below 7 5 g (50%) if there is real doubt about the cause the bone marrow should be examined and the serum iron estimated Not infrequently in such cases these examinations demonstrate that the anaemia is of a different or mixed origin Even if they merely establish that erythropoiesis is micronormoblastic and that the iron stores are exhausted this serves a purpose for it makes patient and doctor more determined upon adequate treatment The marrow picture does also give a certain amount of information regarding the factor causing the anaemia With recent blood loss the marrow tends to be hyperplastic with active erythropoiesis and many phagocytic cells with very chronic haemorrhage or malabsorption it is usually hypoplastic and when there is a large nutritional element giant metamyelocyte formation may be prominent Demonstration that the serum iron is low and the unsaturated iron combining power of the serum is increased also helps to confirm the diagnosis

Oral treatment

Oral treatment should be given whenever possible and other methods only resorted to if this fails or is contra indicated Various ferrous iron preparations are available and in the uncomplicated case they are almost equally effective in equal dosage but in both human cases and animal experiment ferrous sulphate is slightly more irritant than the organic preparations ferrous gluconate fumarate or succinate This is borne out by the fact that deaths from ferrous sulphate poisoning were common when this was the drug of choice but even children who are known to have ingested considerable numbers of ferrous gluconate tablets have recovered Chelated iron preparations do not appear to have any advantage over the other preparations in the uncomplicated case

The most convenient way to give iron is probably as a tablet and 100 mg of elemental iron a day is adequate Ferrous fumarate has the advantage over ferrous gluconate and succinate of not requiring to be coated also only two tablets are needed to provide the daily 100 mg of elemental iron Fluid preparations are more difficult to dispense and the sweetened forms tend to be expensive At this dosage the cost per week at present day prices for tablets would be *Ferrous sulphate 4d fumarate or gluconate 5d* succinate *7d* an amino acid iron chelate *7d* to *1s 4d* and for liquid preparations ferri et ammon citrate *2d* to as high as *4s 8d* for some of the elixirs

Postgastrectomy anaemia

Most cases of iron deficiency following partial gastrectomy respond to ordinary oral iron therapy but in a small proportion this fails completely Parenteral iron produces an immediate improvement but the condition tends to relapse in 18 months to 2 years Recently compounds in which the iron is bound to choline citrate sodium edetate and amino acids have become available These offer a new approach and the latter has proved to be effective in a small group of refractory cases (Hobbs & Discombe 1959)

Anaemia of pregnancy

The treatment of the iron deficiency anaemia of pregnancy is complicated by the fact that during the third trimester there is often a more rapid rise in the plasma volume than in the red cell mass This exaggerates the degree of anaemia and at any one moment it is difficult to evaluate how much is due to dilution and how much to iron deficiency Bone marrow biopsies show that the reserve store of iron is usually lost Normally this iron when mobilized is ample to provide for the increase in the red cell mass and after labour it is returned to the stores At the same time the excess of iron absorbed in the absence of menstruation should cover the 500 mg or so given to the foetus with enough to spare to make good the ordinary blood losses at the time of labour These normal arrangements fail if the woman starts her pregnancy without an adequate store of iron or if the amount of iron available is diminished as a result of a poor diet or malabsorption

At 12 weeks the haemoglobin should be normal as the demands of the foetus have not started In the absence of haemorrhage anaemia at this stage means that the woman had been iron deficient before the pregnancy began and the response to therapy should be prompt and adequate At 32 weeks if the haemoglobin is above 12 g (80%) any drop that there has been can be judged to be mainly due to a discrepancy in the rates of plasma to red cell production If it is below 9 g (60%) and other causes are excluded there is a definite iron deficiency Between 9 and 12 g (60–80%) lies the difficult group Both factors are involved but the degree of participation must vary from case to case In most instances iron deficiency plays an important part (Davis & Jennison 1954) As a working rule no woman should be allowed to go into labour with a haemoglobin level of under 10 5 g (70%) and if possible it should be over 12 g (80%) This ensures that ample iron is available to provide a full store for the child and to prevent the occurrence of severe anaemia through repeated child bearing Conflicting views have been expressed on this subject Sisson &

the cases can be divided into two groups, those in which the cause of the deficiency has been or can be eliminated, and those where the cause is not amenable to treatment In the first group three months of full treatment is probably sufficient, rebuilding of the iron stores being left to iron from dietetic sources If there is any question of the cause persisting the treatment should be continued for longer, and often a maintenance dosage of one week on treatment every month will maintain such a patient in a state of iron sufficiency With severe losses, continued full treatment may be required and may have to be supplemented by other forms of therapy It is particularly important to continue treatment for a consider able period in those individuals showing marked epithelial changes to ensure that the maximum degree of recovery has been achieved, and so prevent the development of post cricoid carcinoma

Parenteral treatment

In iron deficiency complicated by other disorders the treatment must be suitably modified For example with severe gastro intestinal disease oral iron may be contra indicated In such cases and where there is a definite defect in absorption it is justifiable to give parenteral iron Either the saccharated iron complex Ferrivenin is given intravenously or the iron dextran complex Imferon , intra muscularly It is necessary to start such treatment with a small dose and to be extremely careful in treating patients showing allergic disorders particularly asthma or skin diseases The total dose of iron required should be calculated A simple method is to give 250 mg for every 10% (1 5 g) of haemoglobin deficiency plus a further 500–1000 mg to provide a store This dose should not be exceeded to any substantial extent except possibly in cases where there is continued bleeding

The intravenous iron is probably utilized rather more quickly than the intramuscular for even if the lymph drainage is good 20% of the injected mass remains in the tissues for more than 3 weeks and some of it for very much longer The advantage of the intramuscular over the intravenous preparation is that each full dose 5 ml contains 250 mg against 100 mg of iron All the parenteral iron given is available for haemoglobin formation and an average course of 2 25 g costs 20s as Ferrivenin or 40s as 'Imferon This is comparable to the cost of a 50 week course of one of the medium priced oral preparations

(Recent confirmation of the marked carcinogenic hazard of iron dextran in rats and mice (Haddon A and Horning E S *J Nat Cancer Inst* 1960 24 109) suggests that the compound should no longer be used in medical practice until the risks to man have been elucidated Ed)

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Lund (1957) found iron deficiency in the infants of iron deficient mothers Sturgeon (1959) failed to show such a relationship, but had no really anaemic women in his control series, and Hytten & Duncan (1956) considered, on theoretical grounds, that anaemia in the mother was an advantage For the present the middle course should be adopted, that is that true iron deficiency in the mother must be corrected mild degrees should be treated, and any value above 12 g (80%) may be taken to be normal at 32 weeks

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THE PATHOGENESIS AND DIFFERENTIAL DIAGNOSIS OF THE MEGALOBLASTIC ANAEMIAS

RONALD H GIRDWOOD

The occurrence of megaloblastic change in the bone marrow indicates that a patient is suffering from deficiency of vitamin B_{12} or folic acid or both although occasionally, particularly in infants ascorbic acid depletion may play an additional role Conditions in which folic acid deficiency occurs are as follows

- Nutritional megaloblastic anaemia
- Megaloblastic anaemia of infancy
- Idiopathic steatorrhea
- Coeliac disease
- Tropical sprue
- Organic disease of a large part of small intestine
- Resection of a large section of upper small intestine
- Megaloblastic anaemia of pregnancy
- Pregnancy without anaemia
- Hepatic cirrhosis
- Extensive malignant disease chronic infections
- Administration of certain anti malarials
- Administration of folic acid antagonists
- Administration of certain anti con vulsants to a susceptible subject
- Vitamin B_{12} depletion
- A combination of several causes

Vitamin B_{12} depletion may be found in the following conditions

- Nutritional megaloblastic anaemia
- Megaloblastic anaemia of infancy
- Idiopathic steatorrhoea
- Adult coeliac disease
- Tropical sprue
- Organic disease of lower small intestine
- Resection of lower small intestine
- Megaloblastic anaemia of pregnancy
- ?Pregnancy without anaemia
- Hepatic cirrhosis
- Pernicious anaemia
- Total gastrectomy
- Partial gastrectomy
- Gastro enterostomy
- Blind and stagnant loops of small intestine
- Jejunal diverticulosis
- By passing of absorbing surface of ileum
- Diphyllobothrium latum infestation
- A combination of several causes

Since two chemically unrelated articles of the diet are required to prevent megaloblastic anaemia and since there are so many possible causes for the occurrence of the latter it is obvious that there are occasions when it is not easy to establish which of the two possible deficiencies predominates or why it has come about However clinical diagnosis must not be allowed to become too complex Addisonian pernicious anaemia is the type of megaloblastic anaemia most commonly seen in this country To make an

absolutely certain diagnosis of this for research purposes may be difficult, but normally it is enough to do blood counts find megaloblasts in the bone marrow or in the buffy coat of the blood carry out a test meal using the augmented histamine secretion test (Kay 1953) and look for a response to therapy with cyanocobalamin injections Relatively complex investigations are needed in certain forms of megaloblastic anaemia where treatment has already been given or in the pre anaemic state

METHODS OF INVESTIGATION

In the discussion of methods of investigation particular reference will be made to personal experience in the application of these methods in a research laboratory not undertaking routine work Many of the conclusions have already been published and a detailed study of problems of folic acid metabolism and their investigation will appear later (Girdwood 1960*b*)

The serum vitamin B_{12} level

The level of vitamin B_{12} in the serum is estimated microbiologically the test organisms commonly employed being *L leichmannii* or *Euglena gracilis* With *L leichmannii* which was first used successfully by Rosenthal & Sarett (1952) it is necessary to precipitate the serum proteins Various modifications of the method are used by different investigators (e g Spray 1955) but the simplest method is to use Difco B_{12} Assay Medium USP supplied by Messrs Baird and Tatlock London and *L leichmannii* 7830 Details of the method employed by the present author and of reproducibility of results are given elsewhere (Girdwood 1960*a*) but the findings in 833 such assays are shown in Table 1

Table 1 *Numbers of patients with various serum vitamin B_{12} levels* (L leichmannii *no cyanide added*)

	<50	50–129 (μμg/ml)	130+	Range (μμg/ml)	Mean
Controls	—	13	327	110–850	286 1
Pernicious anaemia (untreated)	228	56	3	—	—
Idiopathic steatorrhoea adult coeliac disease and tropical sprue	5	6	52	<50–600	209 5
Total gastrectomy	4	3	1	<50–280	—
Partial gastrectomy	6	4	18	<50–650	196 3
Gastro-enterostomy	2	3	2	<50–310	—
Blind or stagnant loops	5	3	1	<50–300	—
Other disease of small intestine	4	6	8	<50–280	—
Megaloblastic anaemia from anticonvulsants	1	2	5	<50–190	143
Megaloblastic anaemia of pregnancy	1	3	15	<50–450	184·2
Polycythaemia	—	—	39	140–1090	375 1
Chronic myeloid leukaemia	—	—	3	1100–2400	1600
Hepatic cirrhosis	—	—	4	550–980	712 5

With this organism it is not possible to measure with any degree of accuracy levels below 50 $\mu\mu$g/ml and in order to estimate the means of the results in Table 1 readings below this level have been taken to be 50 $\mu\mu$g/ml In some instances the true means will therefore be lower than indicated

These results were obtained without adding potassium cyanide to the sera Some workers add cyanide others do not There is no doubt that the addition of this substance gives higher readings either by liberation of the vitamin from protein compounds or by prevention of its destruction This is demonstrated in Table 2

Table 2 *Serum vitamin B_{12} levels* (L leichmannii) *in fifty patients*

No cyanide added to serum	60–1100 (mean 287) $\mu\mu$g/ml
Cyanide added to serum	120–1860 (mean 560) $\mu\mu$g/ml

The other organism *Euglena gracilis* was first used for serum vitamin B_{12} assays by Ross (1952) and a method involving the use of strain z was described by Hutner Bach & Ross (1956) The method is more sensitive than that with *L leichmannii* and it is not necessary to precipitate the proteins The tubes are placed in a bath with fluorescent strip lights below for about 6 days and the addition of cyanide does not influence the results (Killander, 1957) The medium is not available commercially The problem that I have found in using this method is that the more you dilute the serum the higher are the results (after multiplication by the dilution factor) suggesting that serum also contains inhibitors of the growth of the test organism In Table 3 there are given results already published (Girdwood 1960*a*) of quadruplicate assays in forty patients

Table 3 *Serum vitamin B_{12} levels in forty patients*

L leichmannii (no cyanide)	60–1100 (mean 253 3)
Euglena gracilis (diluted 1 in 50)	60–320 (mean 191 0)
(diluted 1 in 100)	80–920 (mean 250 8)
(diluted 1 in 200)	120–1120 (mean 314 0)

Because of this problem of possible inhibitors the non availability of *Euglena* medium commercially and the 6 day delay in obtaining readings I have in passing the assaying of serum vitamin B_{12} levels from my research laboratory over to a routine laboratory recommended that they should be done by the *L leichmannii* method I have also suggested that 2 μg of potassium cyanide should be added per millilitre of serum before the assay is performed since although the highest result obtained is not necessarily the most physiological one the results by this method are more reproducible than those obtained without cyanide

The absorption of labelled cyanocobalamin

In this country malabsorption of vitamin B_{12} may occur because of lack of intrinsic factor from disease or resection of the absorbing surface in the lower ileum as a result of by passing of this area through a fistula or because bacteria in blind or stagnant loops are depriving the host of the vitamin This can be investigated in several ways

(*a*) *Faecal excretion method*

Here there is measured the percentage output of radioactivity in the faeces after an oral dose of 0 5 or 1 0μg of [58]Cobalt labelled cyanocobalamin (obtainable from the Radiochemical Centre Amersham) This method was first described by Heinle Welch Scharf Meacham & Prusoff (1952) and the findings obtained by various investigators have been discussed by Mollin (1959) It was shown by Mollin Booth & Baker (1957) that to avoid an overlap in the results between pernicious anaemia patients and controls a subcutaneous injection of 0 25 mg of carbachol (carbamyl choline chloride) should be given This appears to stimulate the secretion of intrinsic factor in a few controls who would otherwise absorb less than 50% of a test dose of 0 5–1 0μg My own experience confirms this

The results of 286 tests without carbachol in a variety of diseases are shown in Figs 1–3

(*b*) *Urinary excretion method*

This was introduced by Schilling (1953) There is given a dose of 0 5 or 1 0μg of [58]Cobalt labelled cyanocobalamin by mouth and within 1–3 hr an injection of 1 mg of non labelled cyanocobalamin Callender & Evans (1955) found a range of 15 8–39 6% of the administered radioactivity (mean 25 9%) in the urine in controls after an 0 5μg dose while in pernicious anaemia the figures were nil to 7 5% (mean 2 3%) With a dose of 1μg the range in controls was found by Schilling Clatanoff & Korst (1955) to be 7 0–22 0% (mean 14 2%) whereas in pernicious anaemia the findings were 0 0–2 3% (mean 0 60%) These two groups of workers did not use carbachol My own results in the few patients investigated in this way are given in Table 4

For the purpose of these investigations carbachol was not used but it is advisable to give it as a routine in doing the test It has been shown by Ellenbogen Williams Rabiner & Lichtman (1955) that significantly more radioactivity is recovered if a second injection of non radioactive cyanocobalamin is given 24 hr after the first one and I have confirmed this claim

It is a distinct advantage to have a test involving one, or at most two 24 hr urine collections in place of the ten stool collections of the faecal method

Table 4 *Percentage of radioactivity excreted in the urine after 0 5 µg oral dose of labelled cyanocobalamin with flushing dose*

Normals 14 0 15 2 16 5 22 0 23 0 23 4 26 2
P A Nil nil nil nil 0 41 0 56 2 0 2 1 3 0 4 0 4 9 5 6 5 6 6 0
Stagnant loop Nil 3 3
Idiopathic steatorrhoea 6 5 15 1 16 0 26 0
Jejunal diverticula 0 4
Megaloblastic anaemia from anti-convulsants 19 0
Ileo transverse colostomy 4 7

(c) Blood or plasma radioactivity

Malabsorption of vitamin B_{12} can be shown by estimating the radio activity in the blood after an oral dose (Booth & Mollin 1956) but the labelled cyanocobalamin has to be of very high specific activity

(d) Hepatic uptake method

This was introduced by Glass Boyd Gellin & Stephanson (1954) If labelled cyanocobalamin is given by mouth to control patients, it is absorbed and stored in the liver where it can be estimated by means of a directional scintillation counter placed over surface projections of the organ If there is malabsorption of vitamin B_{12} very little radioactivity will be found over the liver

Correction of malabsorption of labelled cyanocobalamin

If malabsorption of vitamin B_{12} is due to lack of intrinsic factor this will be correctable by an intrinsic factor concentrate prepared from hog stomach

If malabsorption is due to bacteria in blind or stagnant loops depriving the patient of vitamin B_{12} it will frequently be possible to correct this malabsorption temporarily by giving antibiotics by mouth For instance tetracycline is effective if given in a dosage of 0 25 mg q i d for 3 or 4 days Much of the work that I have done in relation to malabsorption in organic disease of the small intestine has been in collaboration with Dr Andrew Doig and the details of this work are published elsewhere (Doig & Girdwood 1960) The results of attempts to correct malabsorption of labelled cyanocobalamin are summarized in Table 5

When intrinsic factor preparations are used it is of practical importance that some batches have been found to inhibit absorption if given in larger amounts than necessary (Glass Boyd Stephanson & Jones 1955) Moreover continued administration of hog intrinsic factor preparations may lead to the development of a state in which they fail to increase absorption

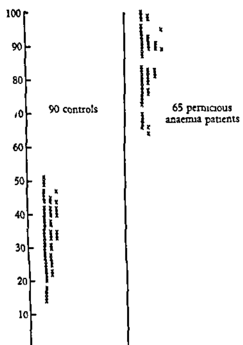


Fig 1

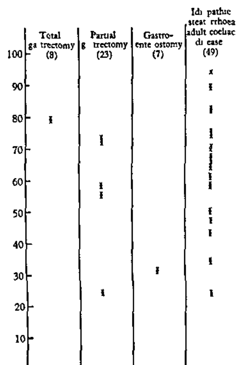


Fig 2

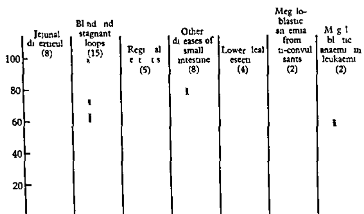


Fig 3

Fig 1–3 Percentage of radioactivity in faeces after 0 5 μg test dose of cobalt labelled cyanocobalamin

Table 5 *Effects of various agents on malabsorption of labelled cyanocobalamin (numbers of patients)*

A Correction of malabsorption		B Failure to correct malabsorption	
By antibiotics		By antibiotics	
Blind or stagnant loops	5	Resection of lower ileum	3
Jejunal diverticula	2	Idiopathic steatorrhoea	2
Oesophago jejunal anastomosis	1	Regional ileitis	2
Partial gastrectomy	1	Ileo transverse colostomy for ileo caecal TB	1
Gastro enterostomy	1	Pernicious anaemia	1
Jejuno transverse colostomy	1		
By intrinsic factor		By intrinsic factor	
Pernicious anaemia	18	Stagnant loop	1
Partial gastrectomy	8	Partial gastrectomy	1
Total gastrectomy	4	Regional ileitis	1
Gastro enterostomy	3	Resection of lower ileum	1
Oesophago jejunal anastomosis	1		
By operation			
Closure of ileo transverse colostomy	3		
Resection of area of jejunal diverticula	1		

(Schwartz Lous & Meulengracht 1957) If antibiotics are given for more than 4 days they may cease to control the bacteria in the gut that are depriving the patient of vitamin B_{12} (Doig & Girdwood 1960)

To be certain that a patient s gastric juice contains intrinsic factor it is necessary to test its ability to promote the absorption of labelled cyanocobalamin in a patient with pernicious anaemia

The serum folic acid level

Unfortunately this cannot be measured relatively easily like the serum vitamin B_1 level Various unsuccessful attempts have been made to develop a suitable method and I have spent quite a lot of time myself in attempting unsuccessfully to do this with *S faecalis* as the test organism Results were unsatisfactory whether the serum was added to culture medium after protein precipitation or aseptically without such manipulation Toennies Usdin & Phillips (1956) and Usdin Phillips & Toennies (1956) found that blood haemolysates would support the growth of *L casei* but attributed this to a number of components that did not include folic acid or folinic acid More recently Baker *et al* (1959) have declared that an *L casei* assay gives a satisfactory indication of the serum folic acid level Working with me in an attempt to develop this method has been Dr Pilar Gomes a World Health Organization Fellow from the Institute of Tropical Medicine Lisbon We have found the organism very difficult to work with and cannot as yet pass any comment on the practical value of this test

Measurement of the urinary output of folic acid after an injected test dose

This does not give much information If a 5 mg test dose of folic acid is given subcutaneously to a patient depleted of the vitamin there may be a diminution in the output in the urine compared with what is found in control patients but sometimes the amount excreted is quite normal

Measurement of the clearance from the blood of folic acid that has been injected intra enously

This test was introduced by Chanarin Mollin & Anderson (1958) and they showed that if 15 μg of folic acid per kilogram of body weight was injected intravenously it was cleared more rapidly than normally in pregnancy megaloblastic anaemia of pregnancy idiopathic steatorrhoea megaloblastic anaemia of leukaemia severe untreated pernicious anaemia and in a few cases of iron deficiency due to chronic haemorrhage I have tried this test in thirty patients and found clearance to be rapid in pregnancy and in disseminated malignant disease Whether increased clearance necessarily means depletion or whether it sometimes indicates increased catabolism of excess folic acid remains to be seen

Measurement of the rise in the serum level of folic acid after an oral test dose

The first report of a folic acid absorption test of this nature was by Denko (1951) Spray & Witts (1952) found flat plasma curves when they gave 1 mg of folic acid to nine patients with untreated pernicious anaemia and five with idiopathic steatorrhoea as compared with twelve normal persons and nine with treated pernicious anaemia My first experiments with such a test were in 1955 (Girdwood 1955) when I gave 5 mg of folic acid and 50 g of glucose to thirteen controls and three with idiopathic steatorrhoea treated with folic acid These three had flat blood sugar curves and flat plasma folic acid curves

Chanarin Anderson & Mollin (1958) gave 3 mg oral test doses to patients first saturated with folic acid and found impaired absorption in idio pathic steatorrhoea pregnancy and megaloblastic anaemia of pregnancy In a subsequent paper (Chanarin MacGibbon O Sullivan & Mollin 1959) they gave 40 μg of folic acid per kilogram of body weight by mouth in pregnancy and measured the rise in the level of folic acid in the serum From this and from studies of the clearance of folic acid given intra venously they concluded that folic acid deficiency is a usual feature in pregnancy and that impaired absorption may be partly responsible but

that unknown factors may be required to precipitate the development of megaloblastic erythropoiesis I feel that further information is required about the metabolism of folic acid in the body a matter of great complexity (Girdwood 1959) before we can be sure of the interpretation of these data

Comparison of the output in the urine of folic acid after injected and oral test doses

It has been suggested (Girdwood, 1953 1956) that a suitable test of folic acid absorption would be to give the subject 5 mg of folic acid sub cutaneously collect the urine for 24 hr, then give 5 mg *orally* collect the urine for 24 hr, and measure the amount of folic acid in each of the 24 hr urine collections The output in the urine is not very different normally whether the folic acid is injected or given by mouth Such a test has been supported by Butterworth Nadel, Perez Santiago Santini & Gardner (1957) and by Cox, Meynell Cooke & Gaddie (1958), but not by Chanarin, Anderson & Mollin (1958) The details are considered elsewhere (Gird wood 1960*b*) and it is sufficient to state here that the test organism is *S faecalis R* and that Difco folic acid assay medium is used

$$\text{The ratio } \frac{\text{Urinary folic acid output after 5 mg oral dose}}{\text{Urinary folic acid output after 5 mg subcutaneous dose}} \times 100$$

is the excretion index From the results of the many tests that have been done I would say that, under the conditions of our laboratory an output of less than 1 5 mg after an oral test dose together with an excretion index of less than 75% indicates malabsorption The results of 524 tests are summarized in Table 6

In Table 7 there are given the numbers of patients in the main groups *investigated who showed abnormal results when the test was done*

There was evidence of malabsorption of folic acid according to the criteria given above in ninety cases of idiopathic steatorrhoea sixteen of adult coeliac disease and eight of tropical sprue Malabsorption was also found in six children with coeliac disease and in six patients with extensive organic disease of the small intestine such as reticulosis or regional jejunitis It occurred temporarily in one patient after resection of a portion of jejunum for diverticulosis (Doig & Girdwood 1960) When the test was first described I suggested that the patients should be saturated with folic acid before it is done by four daily intramuscular injections of 15 mg of folic acid This is sometimes required if the urinary outputs of folic acid after both the injected and the oral doses are low but frequently satura tion is not required Of the 114 adult patients in the sprue group with malabsorption of folic acid twenty two had been treated with folic acid

Table 6 *Results of folic acid absorption tests*

	Number of cases	Mean output of folic acid in urine after 5 mg oral dose (mg)	Range (mg)	Mean excretion index (%)	Range (%)
Hospital controls	220	2.55	1.3–4.4	103.8	66–450
Pernicious anaemia	72	2.19	0.12–3.6	154.5	59–840
Idiopathic steatorrhoea adult coeliac disease and tropical sprue	129	0.608	0.002–2.9	31.9	0.22–135
Coeliac disease in children (active)	8	0.960	0.24–2.3	41.5	7.4–91
Coeliac disease in children (treated)	4	2.06	1.6–2.6	82.5	48–121
Control children	9	2.72	1.7–4.0	122.3	48–370
Total gastrectomy	6	2.38	2.1–2.8	114.0	73–219
Partial gastrectomy	25	2.57	1.3–4.3	130.2	66–436
Gastro-enterostomy	9	2.12	1.6–2.7	135.8	59–392
Blind or stagnant loops	6	2.30	1.7–2.9	109.3	68–308
Jejunal diverticula	4	2.35	1.5–3.6	151.8	94–250
Other disease of small intestine	10	1.53	0.09–2.7	77.1	16–121
Jejunal resection	1	2.40	—	89.0	—
Ileal resection	3	3.27	2.5–4.1	146.0	83–241
Megaloblastic anaemia from anti-convulsants	7	2.49	1.1–4.9	116.3	83–169
Megaloblastic anaemia of pregnancy	11	2.51	1.6–3.4	225.1	67–948

Table 7 *Patients with abnormal results of folic acid excretion tests (numbers of patients)*

	Output less than 1.5 mg after oral dose	Excretion index less than 75%	Both low
Controls (220)	6	32	Nil
Sprue group (129)	119	115	114
Pernicious anaemia (72)	11	9	Nil

before the test was done Occasionally findings suggesting malabsorption have been obtained in control patients but when the tests have been repeated normal results have been obtained

The estimation of formiminoglutamic acid in the urine

In folic acid deficiency formiminoglutamic acid (FIGLU) derived from histidine cannot be converted to glutamic acid because of lack of tetrahydropteroylglutamic acid (THFA) This led Broquist (1956) to investigate the excretion of FIGLU in the urine of children with acute leukaemia during therapy with folic acid antagonists In such patients and in some others with megaloblastic anaemia (Broquist & Luhby 1959) there was more FIGLU in the urine than in normal persons However several workers

myself included did not find this test very helpful or consistently positive in folic acid deficiency Silverman, Gardiner & Condit (1958) and Luhby, Cooperman & Teller (1959) gave a loading dose of histidine before testing the urine for formiminoglutamic acid and this gives promise of being a more satisfactory test of folic acid depletion In Fig 4 there are shown the

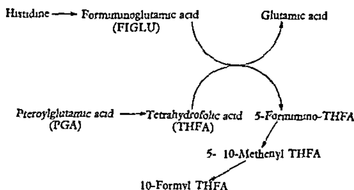


Fig 4 Histidine breakdown

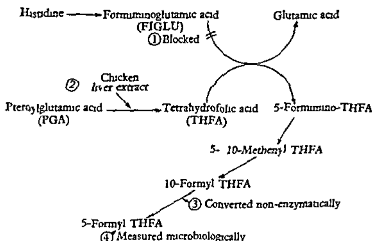
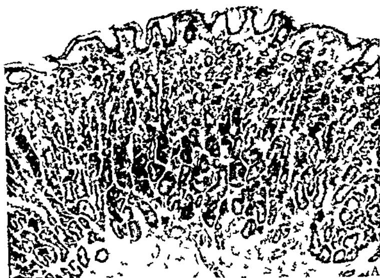


Fig 5 Formiminoglutamic acid estimation

steps involved in the formation of THFA from folic acid (PGA) and the removal of a formimino group from FIGLU by THFA There are various methods to show the presence of FIGLU and the one I am employing at present is that recommended by Silverman Gardiner & Condit (1958) The steps are illustrated in Fig 5

In the absence of THFA glutamic acid formation is blocked (1) In the test a chicken liver extract is prepared in a high speed refrigerated centrifuge and this extract contains enzymes that convert PGA to THFA (2)

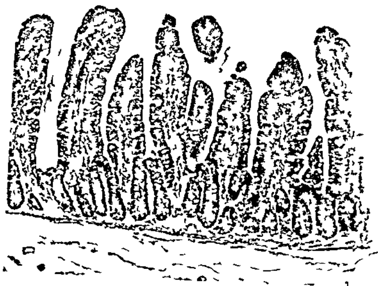


A Normal gastric mucosa



B Gastric mucosa in pernicious anemia

(Face p 52)



A Normal jejunal mucosa



B Jejunal mucosa in idiopathic steatorrhoea

The THFA is formylated enzymatically by FIGLU in the urine and there is formed 10 formyl THFA This is converted non enzymatically to 5 formyl THFA (folinic acid) (3) The latter is then measured micro biologically (4) To carry out the test in this way it is necessary to use small quantities of the expensive triphosphopyridine nucleotide

Gastric and intestinal biopsy

Occasionally it is helpful to do a gastric biopsy as described by Wood, Doig Motteram & Hughes (1949) If the diagnosis of pernicious anaemia is correct one expects to find gastritis or gastric atrophy as shown in Plate 1B

It is also sometimes helpful to do a jejunal biopsy and for this the biopsy capsule described by Crosby & Kugler (1957) has proved very useful in our hands Jejunal biopsy is in its infancy but in idiopathic steatorrhoea one may find thickening and shortening of the villi which become oedema tous and broad ended There is an increase in goblet cells and infiltration of the mucosa by inflammatory cells These features are shown in Plate 2B

THE VALUE OF LABORATORY TESTS IN CONSIDERATION OF THE PATHOGENESIS OF MEGALOBLASTIC ANAEMIA

Addisonian pernicious anaemia

As has already been said this is usually diagnosed easily but there are some patients in whom glossitis or neurological features precede the anaemia and here estimation of the serum vitamin B_{12} level or of the absorption of labelled cyanocobalamin will be helpful Sometimes it will be found that the patient has been receiving folic acid If a patient with Addisonian pernicious anaemia is treated for a period of years with folic acid alone there may develop a state of megaloblastic anaemia that is refractory to folic acid I have seen one such patient (Girdwood 1954) and have witnessed the development of a similar state in a male patient with idiopathic steator rhoea treated with folic acid Presumably he had almost complete mal absorption of folic acid and vitamin B_{12}

In contrast I have seen two patients with Addisonian pernicious anaemia investigated fully by modern methods who did not respond to cyanocobalamin until folic acid was given Presumably their body stores of folic acid were utilized in reactions normally mediated by vitamin B_{12}

Modern investigative methods should be used to replace blood counts in assessing the value of alternative methods of therapy in pernicious anaemia Thus although three patients appeared to be responding reasonably well to a vitamin B_{12} peptide preparation for oral administration over a period of 18 months their serum vitamin B_{12} levels remained in the untreated pernicious anaemia range

Nutritional megaloblastic anaemia

Much remains to be done as regards investigation of the content of vitamin B_{12}, folic acid and related substances in cooked and uncooked foodstuffs but it has been well established that excessive cooking destroys these vitamins In some regions of the world nutritional megaloblastic anaemia appears to be due largely to folic acid depletion However since unlike vitamin B_1, folic acid occurs in green vegetables, one would expect vitamin B_{12} deficiency to be the predominant cause Low serum vitamin B_1 levels have been reported in some patients by Foy Kondi & Manson Bahr (1955) in Africa and by Chatterjea (1958) in India but there is a great need for further investigative work by modern methods In addition to dietetic deficiency of folic acid and vitamin B_{12} and their destruction by cooking other possible factors are malabsorption pregnancy malaria sickle cell disease, small intestinal bacteria deviating vitamin B_{12} from the host and perhaps in some instances the effects of 'daraprim , which although an anti malarial is a folic acid antagonist One would think, too that blood loss from ankylostomiasis must make any deficiency state worse The influence of small intestinal bacteria is uncertain since, although penicillin has haemopoietic activity in some patients with nutritional megaloblastic anaemia (Foy Kondi & Manson Bahr, 1955) it is generally believed that the small intestine normally contains only a few bacteria This might indicate that they are able to colonize areas higher up the small intestine in the tropics, but Nadel & Gardner (1956) found no native flora in the jejunum in sprue patients or controls in Puerto Rico

Megaloblastic anaemia of infancy is a form of nutritional megaloblastic anaemia and it may be associated with kwashiorkor The deficiency is frequently of folic acid but sometimes of vitamin B_{12}

Idiopathic steatorrhoea coeliac disease persisting to adult life, tropical sprue

It is not always easy to be certain with which of these diseases one is dealing but it is too soon to say that all cases of idiopathic steatorrhoea are suffering from coeliac disease that was perhaps undiagnosed in childhood or to rename the condition gluten induced enteropathy The results of various tests given in Tables 1 4 and 7 and in Fig 2 show that there was a low serum vitamin B_{12} level in eleven of sixty three patients malabsorption of labelled cyanocobalamin in twenty nine of fifty three patients and malabsorption of folic acid in 114 of 129 Two had normal absorption of folic acid but malabsorption of vitamin B_{12} while two had normal absorption of both There was an increase in faecal fat (following a collection of at least seven

stools) in 117 of these 129 patients Glucose absorption was abnormal in forty two of forty eight patients and xylose absorption abnormal in sixteen out of twenty

Total gastrectomy, partial gastrectomy and gastroenterostomy

The liver contains about 750–2250μg of vitamin B_{12} while the stores of folic acid are about 3500–7500μg If a total gastrectomy is performed there will be malabsorption of vitamin B_{12} from lack of intrinsic factor but the body stores of the vitamin will maintain the patient s metabolic processes for perhaps three years Then a lowering of the serum vitamin B_{12} level will be followed by megaloblastic anaemia This is inevitable and complex tests are not necessary There is no reason for folic acid depletion to occur other than as a late consequence of vitamin B_1 deficiency and the results of folic acid absorption tests after total gastrectomy shown in Table 6 are normal

The patients investigated after partial gastrectomy or gastro enterostomy were selected because of clinical features suggesting malabsorption After partial gastrectomy particularly if there has been a gastric ulcer or pyloric stenosis megaloblastic anaemia occurs rarely Although steatorrhoea may be a feature there is malabsorption not of folic acid (Table 6) but of vitamin B_{12} (Fig 2) and this can be corrected by intrinsic factor (Table 5) It would appear reasonable to suppose that gastritis destroys the remaining *intrinsic factor* bearing area

It is even rarer for megaloblastic anaemia to develop after gastro enterostomy and it may come many years after the operation Since as is shown in Table 5 there is malabsorption of vitamin B_{12} that can be corrected by *intrinsic factor it is likely that here too gastritis is responsible* for the intrinsic factor defect However in one patient with malabsorption of vitamin B_{12} after gastro enterostomy and one after partial gastrectomy, the defect could be corrected by tetracycline given orally so a blind or stagnant loop may sometimes be responsible

Blind and stagnant loops of small intestine

The results of the various investigations recorded in the preceding tables support the view that bacteria in such loops deprive the patient of vitamin B_{12} but not of folic acid (Doig & Girdwood 1960) Any bacteria that I have obtained at operation from such loops or from jejunal diverticula have been able to remove vitamin B_{12} but not folic acid from culture media The organisms are not peculiar in this respect since most strains of *E coli* can do so Resection of an appreciable segment of lower ileum

results in malabsorption of vitamin B_{12} that cannot be corrected by antibiotics or intrinsic factor, supporting the view that vitamin B_{12} is principally absorbed in the lower ileum.

Organic disease of the small intestine

Since folic acid is normally absorbed in the jejunum and vitamin B_{12} in the lower ileum, a combination of folic acid and vitamin B_{12} absorption tests may give some idea of the extent of involvement of the small intestine in conditions such as regional enteritis.

Deficiency states in pregnancy

Some reference has already been made to this problem in the discussion of tests of folic acid depletion and absorption. There can be little doubt that in the tropics nutritional megaloblastic anaemia occurs in pregnancy and that there may be deficiency both of folic acid and vitamin B_{12}. It has been claimed by several workers (Heinrich & Lahann 1953, Boger, Wright, Beck & Bayne 1956, Okuda, Helliger & Chow 1956) that the serum vitamin B_{12} level falls during the course of pregnancy, sometimes into the pernicious anaemia range, while Baker, Ziffer, Pasher & Sobokta (1958) found that the mother had serum vitamin B_{12} and folic acid levels that were significantly lower than those of the infant at parturition. Sometimes the levels in the mother's blood were only 25% of those in the infant.

These findings and those of Chanarin, MacGibbon, O'Sullivan & Mollin (1959) may indicate that a state of depletion of folic acid and vitamin B_{12} is common in pregnancy. The liver at birth weighs about 120 g and contains about 70 μg of vitamin B_{12} and 400 μg of folic acid substances. No doubt there are great demands for these vitamins by the growing embryo, but one would have thought that the mother's stores would have been more than adequate save in exceptional circumstances. It is strange that megaloblastic anaemia of pregnancy, which in this country responds to folic acid but not usually to cyanocobalamin, sometimes occurs in women who have had normal pregnancies without vomiting or diarrhoea, and whose diet is satisfactory. More rarely pernicious anaemia, idiopathic steatorrhoea or haemolytic anaemia may be complicated by megaloblastic anaemia of pregnancy.

Hepatic cirrhosis

This is rare and little can be said about it. Krasnow, Walsh, Zimmerman & Heller (1957) described seven cases, but the patients were alcohol addicts and may have been suffering from nutritional megaloblastic anaemia. I have never seen a patient with megaloblastic anaemia due to cirrhosis alone.

Megaloblastic anaemia resulting from the administration of folic acid antagonists

Since patients with leukaemia may develop megaloblastic anaemia presumably because of demands by the malignant cells for folic acid it is not surprising that this is more likely to occur in the course of treatment with folic acid antagonists such as 4 aminopteroylglutamic acid The widely used anti malarial pyrimethamine (Daraprim) is a folic acid antagonist and if it is given in a dosage of 25 mg daily for about 50 days instead of in the usual prophylactic dosage of 25 mg once weekly megaloblastic anaemia will frequently occur (Myatt Hernandez & Coatney, 1953)

Megaloblastic anaemia from the taking of anti convulsants

This was first described by Badenoch in 1954 and the first thirty six published cases have been analysed by Stokes & Fortune (1958) The drugs usually involved are phenytoin and primidone The latter has a pyrimidine ring in its structure as has pteroylglutamic acid whereas phenytoin has a five membered hydantoin ring We do not know why these drugs sometimes lead to megaloblastic anaemia and modern methods of investigation have not given the answer

Extensive malignant disease

We have seen that measurement of the urinary output of folic acid after a test dose is not a satisfactory way to show folic acid deficiency because there may be a normal output in a folic acid deficient patient However the test is helpful when it does demonstrate depletion In thirty cases of malignant disease given a test dose of 5 mg subcutaneously the mean output of folic acid in the urine was 1 08 mg (range 0 002–3 46 mg) and this was significantly lower than in control patients In twenty two of the patients with malignant disease the output was less than 1 5 mg However this test has not been employed extensively since the results were normal in some patients with advanced disseminated malignant disease and the information obtained was not helpful in consideration of prognosis In some patients with chronic infection too there appeared to be increased requirements for folic acid

CONCLUSIONS

As a result of newer methods of investigation much has been learned in recent years about the ways in which megaloblastic anaemia may develop Further knowledge is required particularly about changes at the cellular

level occurring for instance when anti convulsant drugs are given, or in pregnancy It is hoped that some of the methods that are being developed to demonstrate folic acid deficiency will prove to be successful

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TRANSFUSION HAZARDS

GEORGE T DISCOMBE

INTRODUCTION

Although blood transfusion saves lives we all know that it has dangers of its own and that from time to time a patient is killed by a transfusion

Fortunately these fatal accidents are rare but for every fatal accident there are probably ten so severe as to make one fear for the patient s life and a thousand which make the patient very uncomfortable but to my surprise I can find but two treatises on transfusion hazards one in French (Andre Dreyfus & Salmon 1956) and one in Hungarian (Hadnagy & Szabo 1957)

Today I want to discuss these accidents and misadventures and to analyse the contributions to them of the clinician the pathologist the nurse the patient the apparatus and the blood

Rather to my own surprise I have decided not to spend much time on the bedside problems of the clinician As a junior he is usually forced to draw conclusions from quite inadequate evidence to take responsibility for a complicated illness in the small hours of the morning with little or no help from his senior colleagues and to account for his actions in the cold light of day to a chief who now has hindsight to guide him

There are however one or two points which are I believe insufficiently stressed First except for the elderly a patient who has bled but needs less than three bottles of blood will usually recover perfectly well without any blood though he may need a plasma expander A patient with acute blood loss who receives but one or two bottles has therefore either been undertreated or overtreated Second blood is still being used avoidably to raise the haemoglobin level of patients to that level which the modern school of anaesthetists considers the minimum for anaesthesia avoidably because in most of these cases the anaemia is due to iron deficiency and the haemoglobin level can be brought up to a tolerable range by oral iron administered in ordinary doses during the waiting period or in appropriate cases by injection of iron preparations such as *Ferrivenin*

It is easy to understand the reluctance of the young anaesthetist to anaesthetize any patient whose condition would be noticeably improved were she to acquire a higher haemoglobin level—for the young anaesthetist is more likely to be attacked in the coroner s court than is the young surgeon and further may get little support from the surgeon who may be seeking a scapegoat Nor will a young anaesthetist care to refuse to anaes

thetize a patient already admitted for operation by a consultant but found to be too anaemic for the anaesthetist's peace of mind. I find it quite impossible to understand or excuse the failure of some surgeons—particularly gynaecologists—to take some action when putting patients on their waiting lists. Many, if not most, gynaecological patients are or have recently been bleeding, and even if it is too much trouble to have haemoglobin estimations done on every such patient, it is much less trouble to prescribe one or two tablets of an iron compound daily until admission. And third, some young men recently qualified will insist on trying to transfuse any patient with severe anaemia, whatever the aetiology—even patients with pernicious anaemia, in whom transfusion is so often fatal. In those rare cases where transfusion is essential we have found that the transfusion of a pint of packed cells with the simultaneous removal of a pint of blood from the other arm permits us to transfuse even patients with pernicious anaemia whose haemoglobin levels are below 20% without detriment.

PERSONALITY AND EMOTION

Most important to my mind are the personalities of the protagonists in transfusion—clinicians, pathologists, patients and nurses.

Clinician and pathologist

When I first had to do with blood transfusion I soon discovered that it was magic—and that the layman attributed all the craft and cunning of Merlin and of Mesmer to the transfusionist. In addition there was a mystique of transfusion noticeable even among my colleagues, and if anything went wrong—and it often did go wrong—there was an emanation from your colleagues which enforced a feeling of guilt in the performer. Just as some wives achieve their ends by phrasing their complaints and demands so as to induce in their husband a feeling of guilty inadequacy, so did some physicians use similar methods to establish their superiority over the transfusionist involved. In general, transfusionists seemed to be either almost incapable of feeling guilty, or felt and behaved like guilty men. It would be interesting if an English medical Mauriac or Bernanos were to consider the ways in which an examination of conscience is utilized by such men to increase their feelings of guilt—or perhaps we might invite William Sansom to meditate on this subject.

Unfortunately if one feels guilty it is impossible to perform a proper self-criticism—and by using that term of neo-Communist jargon I wish to differentiate between self-criticism and the traditional examination of conscience. In a self-criticism the emphasis must be on *actions*. What was

done? In what way did this contribute to the accident? Was there a deviation from the usual procedure? Was the action taken inadequate because of ignorance? It is quite wrong to introduce terms of moral and ethical judgement to say that an action was 'wrong', and that a person 'ought' to have done something Ethical or moral condemnation produces a feeling of guilt, and a man with a load of guilt is unable to amend his ways because he is more than half way to a true depression If we want to prevent future accidents we should take our facts without ethical or moral implications, find out which of them contributed to a particular catastrophe, and then seek to discover how such a catastrophe can be prevented in the future without increasing other dangers

In order to discover what has gone wrong it is essential to establish good communications and this again involves the problem of guilt No house-officer or registrar benefits from being made to feel guilty but everyone benefits from finding out which parts of his treatment or advice were sub optimal and could be bettered—though advice on betterment should usually be implicit rather than explicit Good communications between clinician and laboratory cannot be established unless the desire to impute guilt—which for years has been part of the gamesmanship of seniors—is controlled and overcome Perhaps I may here quote from the current number of the *Lancet* First a reasonable degree of mutual trust must prevail between the surgical and laboratory staff (a state which can be achieved by both sides concentrating on the patient s welfare rather than on each others sensibilities) (Leading article, *Lancet*, 1959 2 1014)

The object of communication is to convey to the appropriate person enough information to make him understand what action is necessary and what action is proposed Two responses are possible—agreement more or less complete followed by appropriate action or disagreement which should be followed by consultation and subsequent agreement No pathologist can safely disagree with a clinician unless he has first made himself familiar with the clinical problem involved and this will need anything from a few words over the telephone with one of the clinical team to a full blown consultation between chiefs registrars and housemen

There was once a gynaecologist who for some reason decided to perform a hysterectomy on a woman of 35 years When this patient was seen some three months after operation she seemed tired and dull so the gynae cologist decided that this was due to post operative anaemia and arranged for a blood transfusion of one pint The pathologist found that the patient s haemoglobin was 101 % or 15 g per 100 ml and very properly protested against the administration of a blood transfusion His pleas went without effect and the pint of blood compatible by all known tests was

duly administered The patient then had one of the most severe types of anaphylactoid reaction her blood pressure fell to unmeasurable levels and she developed anuria—which the same gynaecologist tried to treat by giving large volumes of fluid intravenously The poor oedematous patient then developed a local infection at the site of insertion of the cannula at the ankle then a septic arthritis of the knee and finally after a year in hospital left with an ankylosed knee It will be no surprise to you to learn that the clinician tried to blame the pathologist for providing incompatible blood—which was when I learnt of the incident

Communication is not accomplished merely by speaking or writing to someone Modern techniques are complex and so are the ideas to which they give rise, nobody should be surprised if sometimes a colleague fails to respond to certain ideas and develops an almost paranoid and quite illogical resistance to them Pathologists perhaps more than clinicians are subject to this occupational risk which is getting steadily more and more serious Twenty years ago one had some chance of digesting the two or three advances made each year nowadays there are many made each month and we are afforded even less time to digest them

The pathologist is the man to whom most of these innovations are presented (we are already doing about twenty five or thirty transaminases weekly) and it is his duty to distribute his department s effort according to his estimate of value and need It is obvious that bacteriologist bio chemist haematologist and histopathologist will have very different esti mates of value and need that pathologists of different ages may make different value judgements and that such differences will please some clinicians and anger others but whatever his age or training he must recognize that transfusion needs the most careful organization and super vision for a death from incompatible transfusion is one of the best ways of producing adverse comment in the public press

The pathologist, who is subject to the same moral dangers as the clinician, is in a position to obstruct and make difficult everything which the clinician needs for his own most urgent patient Here above all there is a real hazard in delay and this hazard can be avoided only by the pathologist taking a real interest in clinical medicine and the clinician acquiring an acquaintance with the technical worries of the pathologist In practice this results in the clinician distinguishing very clearly between those patients who need blood rapidly and those who can wait three hours or more and in his using an increasing quantity of plasma substitutes such as dextran to tide over an acute oligaemic episode while the pathologist for his part is ready to go to the wards and discuss the management of the patient in relation to probable demand and probable supply

Patients

Patients contribute very greatly to the development of their own transfusion reactions Gonçalves in 1954 pointed out that his apprehensive and excitable patients all developed rigors and fever during transfusion until he gave them full doses of an opiate—and thereafter reactions became rare I myself advocate promezathine hydrochloride (Phenergan) 50 mg being given by mouth an hour or two before the transfusion as well as being a powerful sedative it is a very satisfactory anti histaminic, so that it abolishes the 2% of allergic reactions (Goldsmith 1957, Stephen, Martin & Bourgeois Gavardin 1955) and it seems to halve the incidence of ordinary febrile reactions I do not think it either safe or logical to add the anti histamine to the blood, for this leaves the patient unprotected at the beginning of the transfusion

There are some diseases which make the patient much more susceptible to severe reactions, and of these the most noteworthy are Crohn s disease and ulcerative colitis In addition to these two conditions many patients with low-grade infections seem to develop fever immediately after a transfusion so that the transfusion is incriminated The reason for these reactions is still obscure but Milgrom & Swierczynska (1956) and Swierczynska (1957) have shown that in prepared animals the injection of erythrocytes coated with certain bacterial extracts causes a severe anaphylactoid reaction whereas the injection of the bacterial extract alone produces few or no symptoms The condition for this reaction is that the animals shall have been sensitized to the strain of bacteria whose extract is used to coat the cells

The patient with ulcerative colitis or Crohn s disease is certainly sensitized to all sorts of bacteria—but where is the bacterial extract to coat the red cells? Must it come from bacteria or can possibly a similar antigenic configuration on a normal human protein cause the response?

However even when patients are given sedative anti histaminics there remain 2–3% of patients who react to almost every transfusion, and not all these have Crohn s disease or ulcerative colitis Sometimes one can avoid transfusion but occasionally it may be life saving In such cases it is worth while protecting the patient with a corticosteroid hormone such as 25–50 mg cortisone injected an hour before transfusion is started or equivalent amounts of the newer steroids or of ACTH (Mainzer 1955) In extreme cases 4–6 mg hydrocortisone per hour given as the free alcohol or the sodium salt of the hemisuccinate will prevent reactions even to gram quantities of protein from a foreign species

However steroids should be avoided wherever possible for even small

doses reduce the endogenous production and may speed collapse while I have encountered a case in which steroids were being used to prevent the symptoms of reactions subsequently shown to be due to an irregular haemolytic antibody

One must remember that the patient depends on successfully maintaining homeostasis and that if very large volumes of banked blood of which the plasma contains a gross excess of potassium and of citrate are transfused he may be unable to metabolize them as fast as he needs Since citrate complexes calcium the citrate tends to potentiate the action of the potassium and a large transfusion may weaken the contractions of the heart and cause a fall in blood pressure instead of the expected rise (Bunker Stetson Coe Grillo & Murphy 1955 Strawitz Howard & Artz 1955 Ivy Greengard Stein Grodins & Dutton 1943 Bruneau & Graham 1943 Cookson Costas Durieux & Bailey 1954 Nakasone Watkins Janeway & Gross 1954 Firt and Hejnal 1957 Wexler *et al* 1949 Ludbrook & Wynn 1958) Injection of calcium gluconate into a *different* limb in a dose of 1–1 5 g for each 500 ml transfused will often restore the blood pressure more quickly than simple transfusion especially after trauma or in the presence of liver disease

But however carefully one tries to avoid citrate intoxication or febrile reactions the most careful precautions are of no avail with the confused uncooperative patient who pulls his drip out, in whom sedatives either increase confusion or send him into a semi coma from which he may not wake Each year the acute patients who get bundled through the wards become more numerous and need more special nursing the permanent shortage of nurses makes it imperative that every means be adopted to allow nurses to attend to their primary duty

Nurses

The nurse is a human being with a mind of her own one no less open to error than any other Here again we need a synonym for error which does not imply moral condemnation—variaction dysreaction or fluff — just as a similar word has been demanded for the occasional unexpected behaviour of an air line pilot Quite apart from the effect of excessive pride which now seems to be less common nurses can do the most absurd things one reputedly responsible staff nurse was instructed to give 60 000 units of heparin divided between two bottles of blood and in error put the whole 60 000 into the first Instead of seeking more expert advice she obtained a large bowl and funnel first boiled them then flamed them by igniting alcohol in them poured the contents of both bottles into the bowl and then returned them to the bottles using the funnel She then left both

bottles on the window sill of a warm room The medical officer did not come to set up the transfusion that night, and the next morning the ward sister recognized the obvious signs of gross bacterial contamination

I have elsewhere commented on other peculiar actions of nurses such as warming blood by immersion for a minute or so in the ward boiler (I had better say at once that none of these examples comes from my own hospital)

THE USE OF FORMS

Between the patient the nurse the clinician and the pathologist there is an obvious web of interest Unfortunately satisfactory intercommunication usually requires that requests and reports be made in writing for everybody has learnt long ago that nobody else s memory is really trustworthy— (Medico legal Report, 1949) and in blood transfusion we must have a reasonable summary of the pertinent history, in a woman a history of previous transfusion the history of her pregnancies and of any infants which may have been afflicted by haemolytic disease of the new born or in a man any history of previous transfusion enough of the present clinical condition must be indicated to enable the laboratory to assess the probable demand—for of course haematemesis from a peptic ulcer has a smaller demand for blood and hence a better prognosis than has one from cirrhosis of the liver

The filling of forms is an exercise which brings out the worst instincts in us all and it is therefore imperative that the forms we design be simple easy to fill up and demand the minimum of information The worst form I know is the standard label and report N B T S 11 for this leaves quite inadequate room for the identification of the patient demands far more information than is usually collected and provides too little space to record it Failure to inform the laboratory of previous transfusions or haemolytic disease especially when transfusion is needed urgently may lead to disaster indeed it may seem a paradox that the more urgent the transfusion the more meticulous in completing the form should be the clinician

A final factor as important in the wards as in the out patient departments and the laboratory is the identification of specimens Inexperienced people do not realize how many specimens may be collected at one time and having collected a blood specimen into a tube will bring it back to the nursing station to be labelled there it may be confused with others

An American study suggested that in an out patient clinic as many as 1 in 400 specimens might be mislabelled and I am inclined to regard this as a conservative estimate I prefer to label the tube take it to the bedside check identification and then collect the blood but even this has led to

errors in identification and some prefer to label the tube at the bedside One must of course remember that some deaf elderly patients will cheerfully assent to everything a doctor says or does so that one must phrase one s questions in such a way that they call for a more complicated answer than yes

I can now abandon the embarrassing subject of variactions fluffs or errors made by the medical or nursing staff It has occupied a third of my time not because I myself do not find it embarrassing but because I firmly believe that failure of communication and blunders (or fluffs) in conduct are responsible for nearly all the serious errors of blood transfusion

STERILITY

The most important factor in keeping blood banks safe is sterility and it is noteworthy that in many countries deaths from infected blood appear to be more numerous than those from other errors associated with blood transfusion In North London the deaths from infected blood seem to be no more than about 1 for every 1 000 000 bottles issued—a remarkable achievement which few other countries can equal—and the rarity of infected blood helps us to forget the meticulous attention to detail which is needed to avoid it

The contamination of blood by bacteria is rarely confined to a single bottle and so one gets occasional outbreaks in which between one and twenty people die quite quickly after transfusion The clinical manifestations are usually a rigor with high fever sometimes falling to subnormal levels sometimes an epileptiform attack with incontinence and always a generalized erythema which may become blotchy and a catastrophic fall in systolic and diastolic blood pressure recovery after the onset may appear to be rapid and satisfactory but the blood pressure does not rise and death usually occurs within a few hours Recovery has sometimes followed treatment with noradrenalin corticosteroids and antibiotics (Braude Williams Siemienski & Murphy 1953) I myself have just had a case in which recovery occurred with no treatment other than the injection of 50 mg promezathine hydrochloride

Bacterial contamination is not important if it occurs within an hour or so of the blood being administered hence the casual manner in which unbanked blood can be treated but if contamination occurs several hours before use with the blood at room temperature or a few days at ice box temperature then multiplication of bacteria will have rendered transfusion dangerous The usual infecting organism is a gram negative rod of the

Pseudomonas or *Achromobacter* genus which grows well though slowly at +4° C, has an optimum at +25° C, but grows slowly or not at all at +37° C (James & Stokes 1957)

Similar organisms are distributed universally—in dust on cotton wool in the drips of a tap in randomly sampled bottles—and the more recent outbreaks seem to have been due to the most absurd sources of contamination One outbreak (McIntegart, 1956) seems to have been due to contamination of the fluid in which were stored the viscose caps which are used to cover the tops of blood bottles these organisms grow easily in chlor xylenols and in aqueous phenol but are inhibited by chlorhexidine (hibitane) so that nowadays the caps as received from the manufacturer are autoclaved in 5% ethylene glycol and stored in 0 1% chlorhexidine for 24 hr before use to remove the glycol (James personal communication)

Another outbreak was caused by using swabs which had not been properly sterilized to apply alcohol to the cap of bottles the swabs were originally properly sterilized in paper bags but one bag had been opened closed and reopened the next day (personal communication—not from the United Kingdom)

A third rather less logical was caused indirectly because a serologist complained that there was never enough blood in the pilot bottles The house physicians who collected the blood decided to fill the pilot bottle first and then transfer the needle to the main bottle for collecting the main bulk for transfusion They did not know that the pilot bottles used were not sterile and were attached *after* the main bottle had been autoclaved (personal communication—not from the United Kingdom)

Elsewhere another technician tapped a bottle seven times before issuing it when its use led to the death of a patient (Haematologist 1958)

Safety is thus attained at the price of eternal vigilance Most of those who use transfusion equipment have no clue how it is prepared and I have great sympathy with any transfusion centre which refuses to accept blood except when collected by its own staff at regular sessions

APPARATUS AND EQUIPMENT

The apparatus used for transfu ion can affect the comfort and even the safety of the patient With red rubber tubing which is used repeatedly it is probably impossible to remove every trace of protein from the inside of the tube so that denatured protein is left thereon and can be removed in small quantities to induce a febrile reaction in the recipient With such tubing also small holes made near the needle in order to inject drugs may not appear until pressure is applied they are of little real but of con

siderable aesthetic importance And finally red rubber always allows something to dissolve out which affects the walls of veins and produces a sterile thrombophlebitis if the infusion or transfusion be allowed to run for much more than 12–18 hr The appearance of this thrombophlebitis depends on many factors but principal among these is the nature of the red rubber employed but, even with the same rubber the production of thrombophlebitis depends on the speed with which the transfused blood is diluted by the recipient s blood and this of course is greater in large proximal veins than in small peripheral ones It follows that transfusions

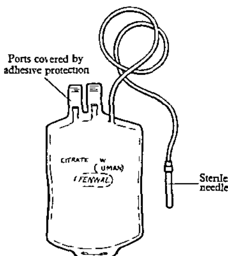


Fig 1 Fenw l plastic blood-collecting pack which contains anti-coagulant but no air and is filled passively A herm t c al of the tube can be obtained by a d electric sealer or by tying a knot in the tube

especially long continued ones are best given into large proximal veins in which the current of blood is considerable and if possible into an arm vein where the hydrostatic pressure is but a few centimetres of water rather than into a leg vein where there may be 25 or 30 cm water pressure or in pregnancy abdominal tumour or ascites much more Until recently *teaching was all the other way cannulae were tied into the smallest* most distal veins in the vain hope that the vein would thrombose only to the next tributary but now people are slowly realizing the logical consequences of the discovery that thrombophlebitis is the result of prolonged exposure of the vein wall to irritants and are tending to run transfusions through needles rather than cannulae and to limit the duration of transfusion into any one vein to 8–10 hr changing regularly from one vein to another

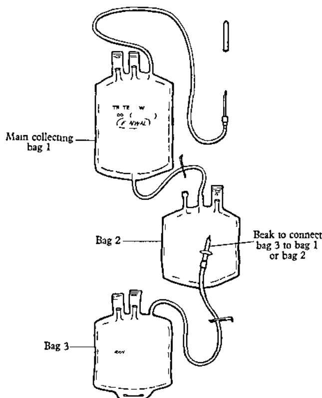


Fig 2 Fenwal separation pack after sedimentation plasma and platelets can be transferred from bag 1 to bag 3 platelets deposited and plasma returned to bag 2

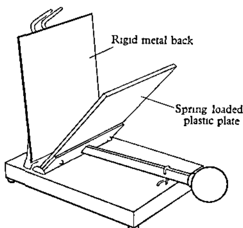


Fig 3 Apparatus for expelling plasma from collecting pack into transfer pack when preparing fresh plasma or concentrated red cell suspension

(Anon 1955, Jones 1957) Polak (1956) thinks that hydrocortisone added to an infusion fluid reduces the likelihood of thrombophlebitis developing

The latest apparatus seems likely to reduce the incidence of thrombo phlebitis even further it is made of a special P V C plastic and is dis posable No irritant extractives are obtained from it so that longer trans fusions may be run but the lumen of the tubing is small the filter area small so that the maximum rate of infusion is appreciably less than with the old apparatus—and of course it is a good bit more expensive than the

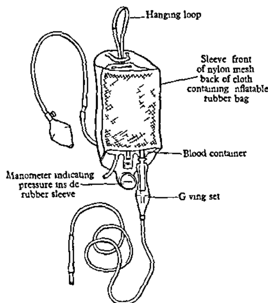


Fig 4 Pressure infuser a blood p ck is held b tween nylon mesh and inflatable sleeve

old but on the whole it seems an improvement and when a plastic con tainer can be introduced as well our transfusion procedure will be notably simplified

The disposable plastic packs made by Fenwal were originally designed for the Boston team working on the Cohn blood fractionator and have been developed extensively I believe they are the best equipment yet made (Gibson 1956 Dudley Richmond McNair Paton & Cumming 1958) The figures show a standard 500 ml pack (Fig 1) a pack for separating cells plasma and platelets in a closed system (Fig 2) a spring loaded press for either separating plasma from cells or speeding up a transfusion (Fig 3) and a pressure device consisting of a mesh pouch containing an inflatable rubber bladder (Fig 4) which will speed up the infusion of blood without

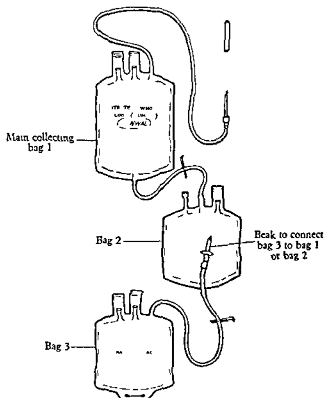


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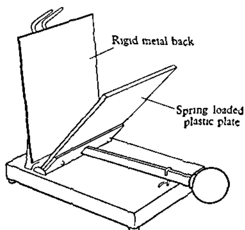


Fig 3 Apparatus for expelling plasma from collecting pack into transfer pack when preparing fresh plasma or concentrated red cell suspension

enough to slow enzyme reactions but not cold enough to damage the cell physically—but if blood be chilled to below 0 56 C the cell wall is damaged by the cold or warmed to over 38° C it is damaged by the heat and then survival is much less Indeed it has been shown that the simple physical process of warming from +4 C to room temperature and cooling again to +4 C is an insult so severe as to reduce the survival time of blood very considerably

During the early part of the last war blood was sometimes transported in uninsulated transport aircraft flying at heights up to 20 000 ft and tempera tures down to twenty degrees below zero centigrade I have heard experts in war medicine discussing whether before they learnt their lesson a particular Air Force lost more men by enemy action than by transfusion with frozen and thawed blood

Similar events rarely occur nowadays but I have met a refrigerator whose temperature regulating control broke down and froze everything in it including the blood and my present blood storage refrigerator is in an unheated outhouse whose temperature in winter may fall well below freezing point To counteract this danger I have had an electric socket fitted inside the refrigerator and in winter we put in some kind of heater ordinary winter weather can be combated quite easily by a 75 W lamp but in severe weather this may have to be increased to 100 W for a 90 ft^3 cabinet

The converse danger of overheating is fortunately rare because the idea of warming blood has gone out of fashion At one time it was the rule that blood should be warmed before being given and there is no doubt that some blood was warmed in most peculiar ways Baker in 1937 described a fatal transfusion of blood which had been maintained at +55 C for several hours and I have met nurses quite capable of doing even more peculiar things

But cells can be destroyed by agents other than physical ones and of these the most important are antibodies to the antigens on the blood cells This is the usual mechanism of an incompatible transfusion and in fact people nowadays tend to equate the term incompatible transfusion with shortened red cell survival resulting from undetected antibodies Unless the term incompatible transfusion is properly defined it leads to mis understanding—most people have a vague idea compo ed partly of trans fusion reactions and partly of presence of antibodies to donor cells For choice the term should be avoided and I would prefer to use the term transfusion reaction for any type of reaction detectable clinically and to qualify this by specifying either the type or the mechanism by which the reaction is mediated

the risks usually associated with the Higginson syringe method which when the vein relaxes, usually projects all the blood into the vein in a few seconds followed by ½ litre of compressed air to kill the patient Of course, by now the compressed air method should be replaced by one of the rotating hand pumps such as that of Martin

Of these plastic packs the platelet pack is already available from Regional Transfusion Centres in this country, and the others can be purchased though at considerable expense Several experimental trials with different plastics have been made, but no other has been generally adopted

Other types of plastic disposable equipment are available—Baxter, Cutter, Abbott, from America and others from Denmark—but I think these are less flexible and less generally useful

However it is obvious that plastic equipment requires complicated machinery and organization to make it properly and it is common knowledge that Fenwal were placed in difficulties by a supplier of plastic sheet who unknown to them changed his plasticizer It is obvious that in Britain we cannot depend on some foreign manufacturer and that we need supplies from at least two different factories before we can abandon our old fashioned equipment So far only one manufacturer is in production

BLOOD AND ITS SURVIVAL ALLERGIC AND HAEMOLYTIC REACTIONS

We now come to the blood itself and must consider the physical conditions for preservation their influence on cell survival and the other factors affecting cell survival when the banked blood is transfused

It is a condition for the safe use of blood that the survival of the donor cells in the recipient shall be of reasonable duration Ideally survival in the recipient should be the same as in the donor and in many cases with very fresh blood this is what actually happens In the absence of antibodies reacting with donor cells even banked blood preserved for a week will show no more than a 10% loss compared with fresh blood

However when blood is stored for longer its life in the recipient diminishes quite rapidly With blood stored in the rather alkaline trisodium citrate 7 days are enough to reduce survival to a few days and even with A C D which helps to maintain cell integrity survival after 28 days storage is short though it can be improved by adding guanosine so that the red cells can survive for a week or more after 42 days storage—though I doubt whether this is useful for I should be very afraid of bacterial contamination (Prankerd 1956)

These figures assume storage under optimal conditions that is cold

elaborated enough antibody to provide a fully haemolytic dose for each cell—usually in 2–3 days For example in a personal case three bottles of group B blood were transfused into a group A recipient After the initial haemolysis and black urine the group B cells persisted in slowly decreasing numbers until the morning of the fourth day when there was a single spike of fever followed by slight clinical jaundice and accompanied by a brisk fall in haemoglobin During the whole of this period anti B could not be detected in the serum, but the direct antiglobulin test was positive in slowly decreasing intensity There was never any reduction in urine volume and in fact the patient was little inconvenienced

It is indeed surprising that in most cases where cells are lysed immediately after transfusion there is little or no interference with urinary secretion When haemoglobinuria does occur it is evanescent and in at least nine cases out of ten the incompatibility is without serious consequences

Other antibodies bring about extravascular destruction in liver or spleen though some haemoglobin perhaps as much as 10% of that transfused may be liberated into the plasma Such cells have half lives of between two and twenty minutes

There is thus clear cut evidence that haemoglobin *per se* has no deleterious effect on the kidneys thus confirming in man what has previously been shown in the experimental animal In some animals the stromata of lysed cells are known to induce a renal shutdown but in many human patients at any rate this seems not to be the case

It seems therefore that some other factor is concerned with the development of the anuria which is the most feared consequence of an incompatible transfusion but which fortunately occurs in no more than five or ten in a hundred of those so unfortunate as to receive one

One important factor in the genesis of anuria is certainly attempts at treatment (Discombe 1954)

My experience is that if a serologically incompatible transfusion has occurred any attempt however well meant to increase urinary output or to speed up the recovery of renal function is far more likely to harm the patient than to help him If the patient is suffering from an acute illness for which the transfusion was ordered then by all means continue to treat that illness by transfusion of compatible blood if necessary but if possible avoid any loading of the patient with electrolytes unless it is quite clear that a lack of electrolytes is prolonging or increasing the illness and above all avoid overloading the patient with water Most patients do best without treatment and the best treatment of impending anuria is probably the withdrawal of all treatment In the few cases where oliguria does occur a normal urinary flow may develop after a few days but in such cases

The common reactions are either allergic or pyrogenic and are rarely of importance they were reviewed earlier There is one type of very serious allergic reaction of which the mechanism is completely obscure—the florid anaphylactoid reaction characterized by fever tachycardia epileptiform seizures incontinence and a catastrophic fall in blood pressure This sometimes accompanies a serological incompatibility—but quite often no serological cause can be found for a severe, or even fatal reaction (Marggraf 1954, Discombe 1952 1954, Hadnagy & Szabo 1959) Sometimes these severe reactions without serological cause may even be accompanied by haemoglobinuria and followed by cortical necrosis of the kidney but this has been recorded mainly in pregnant women near term and it is well known that haemoglobinuria can occur spontaneously under such conditions (Gerisch & Rosner 1956 Aidin 1957) These tragedies are fortunately very rare but may be related to a very acute form of eclampsia

These anaphylactoid reactions are uncommon even when a serologically incompatible transfusion is given—for example the last seen in my hospital was in October 1946 about 40 000 bottles of blood ago since when I have seen some fifteen or twenty serological incompatibilities In retrospect I suspect that some of these anaphylactoid reactions were due to bacterial contamination but there is no doubt that some are not This was the type of reaction which killed a leading Russian experimentalist about thirty years ago in an experiment in which he received only about 50 ml blood I do not know any way of treating them but I suppose noradrenalin and a steroid would give the best chance of survival though the cases in pregnant women which went on to cortical necrosis would presumably be beyond help

However the haemolytic transfusion reactions are mostly due to interaction between an antibody in the recipient which reacts with an antigen in the donor red cells Dr P L Mollison has recently (1959) in his Oliver-Sharpey lectures summarized our knowledge on this subject, to which he has so largely contributed He has shown that haemolytic transfusion reactions can be divided into two groups the first in which transfused red cells are destroyed intravascularly producing high plasma haemoglobin levels and the second in which the cells are sequestered in liver and spleen The first type of reaction is encountered typically in ABO incompatibility and often produces severe symptoms very quickly the plasma being loaded with haemoglobin *within a few seconds of transfusion However even in* this type of reaction large transfusions often absorb all circulating antibody and then cells may even receive on the average a sub haemolytic dose of antibody so that foreign incompatible cells survive in the recipient s circulation for several days to be eliminated only when the recipient has

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seems surprising that the frequency of transfusion hepatitis is no more than 0 16%—for this is the figure obtained by one of the largest and most thorough recent surveys Although no donors with a history of hepatitis are now recruited or used this is not the main factor in maintaining so low a figure for a fall in the incidence of hepatitis due to small pool plasma from 1% to 0 12% occurred spontaneously over a period of a few years before the ban on donors with a history of jaundice was enforced (Ministry of Health 1954 and personal communications) It is true that transfusion jaundice seems to be a severe form of hepatitis and when it does occur leads to prolonged sickness and invalidism—a further argument against the employment of transfusion when it can be avoided

In retrospect then I can sum up my view by saying that the hazards of blood transfusion are considerable A common estimate seems to be that of every 2000 transfused patients one dies from some reason connected with the transfusion I think that in my own practice this may be an overestimate but this will depend on those factors which you will admit to be connected with the transfusion—for example I should not regard under transfusion as connected with transfusion unless the supply of blood had been avoidably delayed. In my own view some accidents are unavoidable—the conveyance of infectious disease particularly hepatitis and brucellosis and probably several virus infections the occurrence of anaphylactoid reactions in the absence of demonstrable antigenic incompatibility serious illness due to the presence of cryophilic bacteria in stored blood which has been properly preserved Others are usually prevented and it is among this group that are found the technical errors which used to play a larger part in the mortality from transfusion than they do now On the part of the clinician in charge of the case he may over transfuse and kill the patient with congestive cardiac failure or fail to recognize the magnitude of the blood lack and fail to administer enough blood to restore the circulation But the most important failures depend on the failure of the therapeutic team as a team on a failure of sympathy between the man who carries clinical responsibility and the man who selects blood for transfusion so that it carries the minimum of risk and gives the maximum of benefit

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it is wise to start the recipient on the Bull regimen or its more modern modifications as soon as reduction in renal secretion is suspected or apparent

CONVEYANCE OF DISEASE

The last and in some areas the most important hazard is the direct conveyance of disease from donor to recipient and this presumes a bacteriaemia or viraemia in the donor We know little of the conveyance of the common virus diseases but by analogy with the experimental virus infections in animals we may assume that viraemia occurs early in the disease and may be marked before the patient develops severe symptoms It therefore seems improbable that we shall be able to prevent such infections but so far there is no evidence that they are frequent

In the past the commonest and most feared of transfusion infections was syphilis but since the recent spectacular reduction in syphilization this risk has become very slight in Britain though still common elsewhere In Britain the rule by which voluntary blood donors receive no monetary reward is a useful precaution against such infections for the voluntary donor is usually conscientious and less likely than most to indulge in sexual promiscuity In West Africa the positive Wassermann reactions are very often biological false positive reactions and it seems possible that this is due to the West African having an inborn tendency to maintain a higher level of gamma globulins than does the European

In some parts of the world malaria is a considerable risk In England today there are enough donors to enable us to reject all donors with a past history of malaria but this is not the case everywhere and in some parts of the world it is impossible In some areas the immune native or old resident may tolerate without a tremor blood which would induce an overt attack of malaria in a foreigner and in such areas non immunes (and perhaps immunes also) need a preventive course after transfusion

Many other diseases can be communicated during the bacteriaemic phase but such accidents appear to be rare Dr Wood drew attention in 1955 to the dangers of conveying undulant fever in areas where it is endemic

However in Britain today the most serious of these infections is hepatitis It seems clear that patients who have suffered from infective hepatitis may carry and communicate the causative virus for years after apparent recovery and that volumes as small as 0 02 ml serum or blood of these carriers (and perhaps a hundredth of this volume) can convey the disease When to this information is added our knowledge that many cases of hepatitis are not recognized because they never become jaundiced it

HAEMOGLOBIN VARIANTS

J C WHITE

Haemoglobin is an outstanding member of the important class of conjugated proteins the haem proteins The members of this class enter into a wide diversity of reactions in the living organism concerned with oxidation reduction and oxygen transporting mechanisms All possess the common structural feature of iron porphyrin prosthetic groups united with protein variation in the nature of the haem occurs (e g cytochrome) but protohaem (ferro or ferriprotoporphyrin IX) is common (haemoglobin catalase horseradish peroxidase) The nature of the protein and of the linkages to the prosthetic group are of great importance in determining the type of activity of these proteins In all higher animals and man haemoglobin is concerned principally with oxygen transport this property being determined largely by the co ordination of ferrous iron in the proto porphyrin molecule and the nature of the linkage of the four haem groups to the surface of the globin

Haemoglobins are of extremely widespread occurrence in nature and are not confined to the red cells of higher animals Examples of its occurrence may be cited in the root nodules of leguminous plants dissolved in the blood fluid of *Daphnia* in yeasts moulds and protozoa

It is not surprising that the globin moiety varies in different haemoglobins the isoelectric point and amino acid composition varying from species to species and the molecular weight being very high in haemoglobin from some invertebrates The haem does not vary in structure Within single species variants of haemoglobin may occur which differ in structure and properties Formerly the best characterized intra species variants were the adult and foetal forms of haemoglobin found in man and many animals

The discovery that sickle cell haemoglobin differs from normal adult human haemoglobin that it is formed under genetic control and that its presence is related to the clinical manifestations of sickle cell disease has stimulated an enormous volume of work in this field Pauling Itano Singer & Wells (1949) advanced the concept of a molecular disease in such conditions as sickle cell anaemia variation in the structure of a normal protein at the molecular level leading to functional disturbance

In recent years many variants of human haemoglobin have been discovered by the application of suitable physico chemical techniques and a good deal is known about the genetic control of their production Very

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recently, outstanding advances have been made in the understanding of the chemical and molecular structure of a number of these variants and an important branch of human biochemical genetics has been illuminated

Perhaps one may quote Tennyson in saying

> Yet all experience is an arch wherethro
> Gleams that untravell d world whose margin fades
> For ever and for ever when I move

Ulysses was despondent at inactivity when he said these lines but there can be no despondency in surveying the amazing advances made in the study of haemoglobin Many tantalizing problems remain but it is clear that their solution will be steadily achieved

HAEMOGLOBIN BIOSYNTHESIS

The questions of haemoglobin biosynthesis and of genetic variations in the molecular structure of haemoglobin are of great interest and importance It is now realized how widespread is the occurrence of sickle cell disease thalassaemia and conditions due to the genetic interaction of these together or with genes for other abnormal haemoglobins (e g Hb S and C in West Africa, thalassaemia and Hb E in the East) Further very rare metabolic disorders often familial such as the porphyrias can now be interpreted at least in part in terms of disturbed production of the pre cursors required for haem formation Fundamental work in a number of originally disconnected fields is now beginning to illuminate a variety of human disorders The general problem of protein biosynthesis has advanced rapidly in recent years and the relationship of the nucleic acids to this process has attracted considerable attention The study of haemo globin formation in the red cell has proved a very profitable subject for such studies and at the same time the formation of the haem portion of this conjugated protein from very simple precursors has been explained to a great extent

Nucleic acids cell growth and protein synthesis

The existence of the nucleic acids has been known since the work of Miescher in the seventies of the last century followed by that of Altmann Kossel Neumann Levine Jones Hammarsten and many others Similar phosphorus containing organic acids had been obtained from plant and animal tissues exemplified by the products from yeast and from the nuclei of calf thymus lymphocytes but whereas the former contained *d* ribose nucleotides of adenine guanine cystosine and uracil the latter contained deoxyribose nucleotides of the same bases except that thymine was found

instead of uracil Subsequently the use of the Feulgen reaction as a cyto chemical test for deoxyribonucleic acid (DNA) and the isolation of pentose nucleic acids from animal tissues indicated that both acids were characteristic constituents of both plant and animal cells and specific enzymes degrading both forms were isolated from both sources

The pioneer work of Caspersson in Sweden and Brachet in Belgium indicated not only the cytochemical distribution of these substances within cells but also their important connexion with protein synthesis and cellular multiplication Whereas DNA was confined to the chromatin of the nucleus ribosenucleic acid (RNA) was found in both the cytoplasm and in nuclear structures such as the nucleolus and was particularly abundant in young and growing cells Caspersson particularly stressed the controlling role of DNA over the hereditary transmission of genetic characters from cell to cell and also the control of protein synthesis in nucleus and cyto plasm Brachet also stressed the role of nucleic acids in protein synthesis but considered that RNA located in the small cytoplasmic particles was involved particularly The extraordinary two stranded helical molecular structure of DNA has been elucidated by Watson & Crick, and by Wilkins and co workers since 1953 and provides a basis for understanding the necessary specificity of DNA as the hereditary determinant in the gene studies in the field of bacterial and mould biochemical genetics are particularly rewarding at present The electron microscopy of ultra thin tissue sections has indicated that the cytoplasmic RNA is largely localized in small particles attached to double lamellar membranes the endoplasmic reticulum and biochemical studies in isolated preparations of particles provide models in which protein synthesis can be studied and it has been found that newly formed protein built up rapidly from free amino acids with the participation of adenosine triphosphate is intimately associated with the ribonucleoprotein of the microsomes

Haemoglobin synthesis in nucleated red cells and reticulocytes

Shemin and Rittenberg (1946) first threw light on the nature of haemoglobin biosynthesis in 1946 showing that ^{15}N labelled glycine was incorporated into the haem of newly formed red cells after administration to a normal man and that the labelled haemoglobin thereafter circulated in the cells for the duration of their mean normal life span of 120 days Altman and others found that glycine labelled by ^{14}C at the α position was incorporated into both the globin and the porphyrin of the haemoglobin The micro spectrophotometric studies on bone marrow cells by Thorell indicated the close connexion between a high RNA content of the early erythroblasts and subsequent haemoglobin formation though he considered that the

haemoglobin did not form until after the completion of synthesis of a pool of globin this latter point is still uncertain, some workers finding that haem and globin are formed in parallel

Although most cells form protein in the presence of both nucleic acids studies on experimentally enucleated cells such as amoebae and algae show that the RNA is more immediately connected with the process The reticulocyte provides a most striking example of this, since a limited but definite degree of haemoglobin synthesis can occur in this non nucleated cell which is still equipped with part of the actively respiring ribonucleo protein rich cytoplasm of the preceding nucleated erythroblast stages London, Shemin, Rittenberg and co workers have shown that not only are nucleated duck erythrocytes capable of haemoglobin synthesis *in vitro* but also the blood in rabbits after haemorrhage and in some human anaemias, such as sickle cell anaemia and some cases of pernicious anaemia In these cases the proportion of reticulocytes is high although in other anaemias with reticulocytosis synthesis may be less active Boorsook and colleagues Holloway and Ripley and Koritz and Chantrenne have shown that amino acids are actively incorporated into the haemoglobin and that the activity of the process is proportional to the RNA content of the reticulocytes

Haemoglobin biosynthesis

Many of the details of the pathway of haem biosynthesis have been elucidated through the work of Shemin and co workers in the United States and of Rimington and Neuberger and their co workers in this country The initial stage involves a condensation of succinyl coenzyme A with a glycine pyridoxal phosphate derivative to yield δ amino laevulinic acid and since the chief energy requirements for porphyrin biosynthesis are involved the cellular control of the pace of formation may operate here Subsequently δ amino laevulinic acid is condensed to the monopyrrole porphobilinogen, and four molecules of the latter condense to form the porphyrin skeleton This probably exists as the porphyrinogen decarboxy lation from uro to coproporphyrinogen and finally to protoporphyrin occurring ferrous iron uniting with the latter to yield haem The whole process appears to be under the control of a series of enzymes and an inhibitor may regulate the rate of the initial stage The latter certainly operates in some experimental systems and Shemin and co workers suggest that decreased activity of the inhibitor may be responsible in human acute porphyria rather than a metabolic block due to deficiency of an enzyme

London and co workers (1958) have shown that the rates of incorporation of ^{14}C-labelled glycine into haem and globin respectively are roughly parallel

in *in vitro* preparations of nucleated duck red cells rabbit bone marrow and rabbit reticulocytes In the latter two preparations the rate of incorporation declines markedly from the younger to the older erythroblast stages and finally to the reticulocyte although the ratio of haem to globin activity remains about unity A number of factors have been found to operate in the cultures however which will markedly dissociate the two processes It may be expected that much light will be thrown on obscure human anaemias involving defective haemoglobin formation from this type of intimate study of the biochemistry of the process

ABNORMAL DERIVATIVES OF HAEMOGLOBIN IN THE RED CELL

Quite apart from the genetically determined structural variants in haemoglobin that depend upon slight differences in the protein moiety and which all appear to be efficient oxygen carrying pigments several modifications of haemoglobin may occur in which the haem group (and sometimes also the globin) and the oxygen capacity of the blood is reduced with resultant cyanosis These may result entirely from the effects of toxic chemicals (sulphaemoglobinaemia and most instances of Heinz body anaemia) or from either toxic or genetic factors (the methaemoglobinaemias)

The outstanding function of intracorpuscular haemoglobin is the transport of oxygen from the lungs to the tissues In addition it plays a part in the transport of carbon dioxide from the tissues to the lungs and in maintaining a stable hydrogen ion concentration of the blood

The ability of haemoglobin to transport oxygen depends upon the ability of the weak acid haemoglobin to form a loose molecular compound with oxygen the resulting oxyhaemoglobin is a slightly stronger acid at physiological pH but readily dissociates on reducing the partial pressure of oxygen $Hb+O_2 \rightleftharpoons HbO$ Under favourable conditions as within the intact red cells the reversible oxygenation is effected without any significant oxidation to methaemoglobin (ferrihaemoglobin) If solutions of haemoglobin are exposed to varying partial pressures of oxygen which are then plotted against the resulting degrees of saturation of the haemoglobin with oxygen oxygen dissociation curves are obtained These are based upon the form of a rectangular hyperbola but a variety of conditions result in the curve assuming a more sigmoid form This is notably so with intact red cells and is advantageous under physiological conditions in that the oxygen is more readily given up to tissues at low oxygen tension

Haldane & Smith (1897) observed that miners could work with a simple anaemia of less than 50% Hb but reduction of the oxygen capacity to the

equivalent level by carbon monoxide poisoning often produced collapse The dissociation curves for both oxygen and carbon monoxide have the same sigmoid form though the affinity for CO is far greater, but the curve for oxyhaemoglobin becomes less sigmoid in the presence of carboxy haemoglobin Roughton & Darling (1944) showed that the curve moves progressively to the left when the percentage oxygen saturation is plotted against partial pressure of oxygen in the presence of increasing percentages of carboxyhaemoglobin

Similar observations have been made by Darling & Roughton (1942) in the case of mixtures of methaemoglobin and ordinary haemoglobin (ferro haemoglobin) the shift to the left increasing with increasing percentage of methaemoglobin This effect on the oxygen dissociation curve has been confirmed by Gibson & Harrison (1947) and Baikie & Valtis (1954) in idiopathic methaemoglobinaemia The tendency to tissue anoxia in the case of methaemoglobinaemias is important clinically and may also obtain in sulphaemoglobinaemia where cyanosis may be very marked in the presence of only 10–15% of the abnormal pigment

Methaemoglobinaemia

Methaemoglobinaemia due to toxic chemicals (aromatic nitro compounds many oxidizing agents etc) and drugs (e g sulphonamides) is fairly common in exposed individuals The amount of abnormal pigment may vary from about 5% (the limit of effective spectroscopic detection) to more than 30% of the total intracorpuscular pigment Once the individual is removed from exposure to the offending substance the methaemo globinaemia and cyanosis rapidly disappear This is because the red cell is normally equipped with a mechanism for the reduction of methaemoglobin which continuously forms by the auto oxidation of haemoglobin

The reduction of methaemoglobin is linked with the glycolytic mechan isms present in the red cell and in particular it appears to be connected with the conversion of lactate to pyruvate in the presence of co enzyme factor 1 (diphosphopyridine dinucleotide) (Gibson 1948) A flavoprotein methaemoglobin reductase (Altman 1954) is also active and ascorbic acid and reduced glutathione may further limit the accumulation of excess of methaemoglobin in the circulating red cell even when the individual is exposed to active oxidizing agents Under normal conditions the efficiency of these reducing systems is such that only traces of methaemoglobin can occur in the cell

A rare form of congenital methaemoglobinaemia is present from birth with up to 35% of the total haemoglobin in the form of methaemoglobin and consequent lifelong cyanosis of the individual This abnormality

appears to be a genetically determined defect in the methaemoglobin reducing systems of the red cell and may involve an absence or defective formation of co enzyme factor I (Gibson 1948) Ascorbic acid given by mouth to these subjects is effective in reducing the amount of methaemoglobin present and the results of such treatment are shown in Fig 1 (the reduction in reticulocyte levels and secondary polycythaemia during the period of treatment is noteworthy) Methylene blue orally or intravenously is also effective in temporarily reducing the methaemoglobin (King White & Gilchrist 1947) and Gibson (1948) has produced evidence that it acts

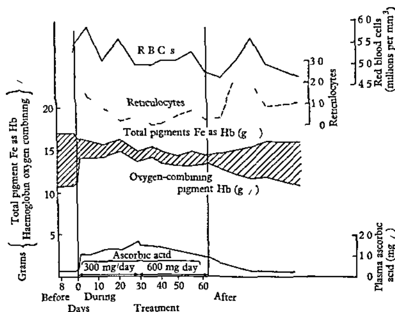


Fig 1 Effects of o al dministration of ascorb c cid on a case of methaemoglobinaemia (King White & Gilchrist 1947)

as an intermediate hydrogen carrier reacting rapidly with both reduced triphosphopyridine nucleotide (co enzyme factor II) and methaemoglobin

Although the oxygen capacity of the blood is much reduced in congenital methaemoglobinaemia physical development and capacity for exercise are not necessarily reduced A young woman with this condition presented herself for ascorbic acid therapy only prior to going dancing and then for the cosmetic effects rather than for any increased exercise tolerance! However some cases are associated with mental defect from infancy though it is not yet clear whether this is entirely due to oxygen lack *in utero* and in early infancy The inheritance of the condition appears to be mendelian

equivalent level by carbon monoxide poisoning often produced collapse The dissociation curves for both oxygen and carbon monoxide have the same sigmoid form though the affinity for CO is far greater, but the curve for oxyhaemoglobin becomes less sigmoid in the presence of carboxy haemoglobin Roughton & Darling (1944) showed that the curve moves progressively to the left when the percentage oxygen saturation is plotted against partial pressure of oxygen in the presence of increasing percentages of carboxyhaemoglobin

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A rare form of congenital methaemoglobinaemia is present from birth with up to 35% of the total haemoglobin in the form of methaemoglobin and consequent lifelong cyanosis of the individual This abnormality

Although Heinz bodies are commonest after exposure to various chemical substances they may occasionally occur as a congenital phenomenon without obvious cause but perhaps related to the presence of an abnormal metabolite or in association with congenital absence of the spleen (Putscher & Marion 1955) The spleen is active in removing cells containing the inclusions, and splenectomized individuals readily form Heinz bodies on exposure to drugs such as phenacetin (Selwyn 1955)

A great deal of work has been done on the nature of Heinz bodies and most workers agree that they contain various structural components of the red cell but mainly consist of denatured and degraded haemoglobin Beaven & White (1954) produced evidence that acetylphenylhydrazide is oxidized to benzene and nitrogen by air in the presence of haemoglobin which acts as the last link in the utilization of molecular oxygen and is itself reduced and then undergoes secondary degradation to insoluble denatured globin and various green pigments by alterations in the haem groups The reaction is entirely aerobic In the intact red cell defective oxygenation and active glycolysis reduce the Heinz body producing activity of acetylphenylhydrazide

Important information has recently been obtained on the nature of drug induced acute haemolytic anaemias associated with Heinz body production Although drugs such as the anti malarial primaquin are harmless to most people certain individuals are sensitive and suffer acute haemolytic anaemia after exposure Sensitivity is particularly high among certain negro racial groups particularly American negroes The work of Beutler and associates has revealed that the red cells of sensitive individuals show a frequently low glutathione content that Heinz bodies form in much greater numbers than with normal cells on incubation with acetylphenylhydrazide and that such incubated cells have a far lower content of reduced glutathione than normal control cells (glutathione stability test) This behaviour is related to a low content of glucose 6 phosphate dehydrogenase in the sensitive cells and may be the primary genetic defect in the sensitive individuals and is found in some 10% of American negroes in cases of favism and in some cases of drug induced haemolytic anaemias The subject has recently been fully reviewed by Beutler (1959)

THE HAEMOGLOBIN MOLECULE

Haemoglobin belongs to the class of globular proteins It has a molecular weight of 67 000 and data from the physical properties and the crystallo graphic studies of Perutz (1958) have indicated that the molecule is roughly spheroidal of the dimensions $55 \times 55 \times 70$ Å with four polypeptide

recessive, and it may be common in some in bred populations (Coudounis 1957, Scott & Hoskins, 1958) A family studied in this country presented normal parents and first born child whilst the three younger children all had methaemoglobinaemia from birth and mental defect (Worster Drought White & Sargent, 1953)

A further rare form of congenital methaemoglobinaemia and cyanosis has been described by Horlein & Weber (1948) and Gerald Cook & Diamond (1957) Here, the abnormality appears to reside in the globin moiety, and to be inherited like other true haemoglobinopathies as a mendelian dominant characteristic The reductive mechanisms of the red cell are not involved (Rossie-Fanelli Antonini & Mondovi 1957) The nature of the haem protein attachments in the abnormal Hb M seem to be such that some react normally, others abnormally

Sulphaemoglobinaemia

Sulphaemoglobin is another abnormal pigment which may occur in the red cells after exposure to various chemical substances particularly aromatic compounds and derivatives of phenacetin Hydrogen sulphide is also required for its formation and in life is probably absorbed from the in testinal tract Analgesics containing phenacetin may be taken in large amounts and for long periods in such long standing painful conditions as rheumatic disorders with frequent occurrence of cyanosis due to sulphae moglobin The amount present usually varies from 5 to 20% of the total blood pigment Unlike methaemoglobin sulphaemoglobin cannot be reconverted to functioning haemoglobin and it is eliminated only by the cells containing it reaching the end of their life span Jope (1946) showed that when healthy individuals developing cyanosis on contact with trinitro toluene were removed from exposure those with methaemoglobinaemia lost all the abnormal pigment in a few days whereas the disappearance of sulphaemoglobin proceeded in linear fashion corresponding to the life span of the red cells of 100–120 days

The Heinz body abnormality of red cells

The occurrence of Heinz Ehrlich bodies as a degenerative phenomenon has long been known particularly after exposure to phenylhydrazine derivatives, or a host of toxic chemical and therapeutic substances (Webster 1949 Fertman & Fertman 1955) Many of these substances are also effective in producing methaemoglobinaemia but the presence of this pigment does not seem to be closely related to formation of the Heinz bodies (Beaven & White 1954) or to the increased susceptibility to haemolysis of cells con taining the inclusions (Beutler 1959)

HbO or the product Over a certain pH range and with controlled alkali protein ratio the reaction is kinetically first order so that a plot of log (unchanged HbO) versus time is linear with slope proportional to the first order reaction constant (K_1) The time for half reaction i e 50% conversion *is also simply related to* K_1 *Either* K_1 *or* $t\frac{1}{2}$ *can thus be used to* characterize the reaction At 20 C and pH 12 8 ($N/25$ alkali) the values of $t\frac{1}{2}$ for Hb A and Hb F are about 11 and 1030 sec respectively corresponding to K_1 values of 0 063 and 0 0067 sec^{-1} respectively It can easily be shown from these figures that the 1 min reaction time used in the Singer residue method corresponds to about 99% conversion of Hb A and to less than 4% conversion of Hb F into alkaline haematin

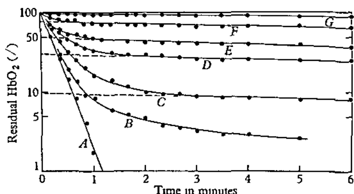


Fig 2 Alkaline denaturation rate plots of mixtures of adult and foetal oxyhaemoglobin Hb F content per cent (*A*) Nil (*B*) 5 (*C*) 10 (*D*) 30 (*E*) 50 (*F*) 80 (cord blood) (*G*) 100 (dialysed residue from partial alkali denaturation of cord blood) (Beaven Ellis & White 1960)

When both Hb A and Hb F are present the plot of log (unchanged HbO_2) versus time is not entirely linear the initial section of high slope corresponding to the conversion of the Hb A component changes smoothly into a final essentially linear section corresponding to the slower reaction of the Hb F This linear portion can be extrapolated to zero time to give the proportion of Hb F in the sample (Fig 2) with reasonable precision In practice the method is reliable down to about 10% Hb F below this level the observations required to establish the linear part of the plot involve measurements of small changes in absorbance over long periods of time and may be subject to instrumental errors etc

Singer Chernoff & Singer (1951) allowed an alkaline solution of HbO_2 of specified concentration and pH to react for exactly 1 min at room temperature the reaction mixture was then neutralized the denaturation product (alkaline haematin) precipitated and the proportion of unchanged

chains internally and four haem groups at the surface Crystallographically, the molecule possesses a dyad axis and it consists of two identical halves Each of these halves is itself made up of the two different α and β poly peptide chains *

The solubility, spectroscopic properties and other physico chemical features have been discussed in detail recently by Beaven & Gratzer (1959) and further reference will be made to some of these subsequently

The physico chemical properties of the haemoglobins of different animal species often differ in slight but significant degrees due to variation in the globin structure In man the foetal and adult forms of normal haemo globin differ in fundamental respects and this pair of pigments was the most extensively studied of the human haemoglobins until recently

FOETAL HAEMOGLOBIN

For many years foetal haemoglobin (Hb F) has been distinguished from the adult type (Hb A) by its greater resistance towards alkali denaturation (Korber 1866) These two closely related proteins differ in many physical properties (for discussion and literature see White & Beaven 1954 1959 Itano, 1957) and some of these form the basis for the estimation of Hb F Differences in the fine structure of the ultra violet absorption spectrum of the globin moiety (Jope 1949) have been utilized Recent techniques of chromatography on resin columns (Prins & Huisman, 1955 Huisman & Prins 1955) and electrophoresis on agar gel (Robinson, Robson Harrison & Zuelzer 1957) also distinguish Hb F from Hb A and from other haemo globin variants However the reaction with alkali remains the most widely applied in practice

QUANTITATIVE ESTIMATION OF HUMAN Hb F

(a) *Alkali denaturation methods*

Since its introduction by Brinkman & Jonxis the alkali denaturation rate method has been widely used though not to the same extent as the more recent Singer 1 min residue method (for references see White & Beaven 1954 Itano Bergren & Sturgeon 1956) A most recent comparison of the two methods has been made by Jonxis and Huisman who concluded that at high levels of Hb-F 1 min values are about 10% low while the denatura tion rate method is inaccurate below 10% Hb F

The conversion of HbO into alkaline haematin is accompanied by a colour change from red to brown which can readily be followed spectro photometrically or colorimetrically at wavelengths appropriate to either

* See below p 105

HbO or the product Over a certain pH range and with controlled alkali protein ratio the reaction is kinetically first order so that a plot of log (unchanged HbO) versus time is linear with slope proportional to the first order reaction constant (K_1) The time for half reaction i e 50% con version is also simply related to K_1 Either K_1 or $t^{\frac{1}{2}}$ can thus be used to characterize the reaction At 20 C and pH 12 8 (N/25 alkali) the values of $t^{\frac{1}{2}}$ *for Hb A and Hb F are about 11 and 1030 sec respectively cor* responding to K_1 values of 0 063 and 0 0067 sec^{-1} respectively It can easily be shown from these figures that the 1 min reaction time used in the Singer residue method corresponds to about 99% conversion of Hb A and to less than 4% conversion of Hb F into alkaline haematin

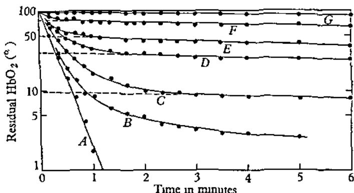


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HbO remaining in solution estimated and reported as Hb F They found normal adult bloods invariably gave 1 min residues in the range of 0 5–1 7% and therefore recommended that values below 2 0% should not be regarded as certain evidence for the presence of Hb F This proviso has not always been applied by users of the Singer 1 min residue method and in consequence many interesting reports of the occurrence of Hb F below the 5% level are not entirely reliable At higher levels above 10% Hb F Singer 1 min residue figures are much more reliable *

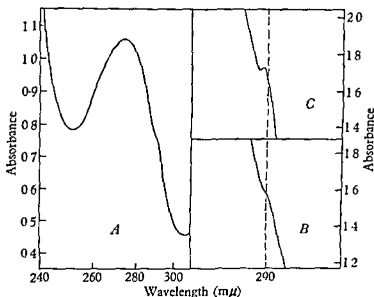


Fig 3 Spectroscopic difference between human adult and foetal haemoglobins *A* absorption spectrum of adult carboxyhaemoglobin (globin absorption band) over wave length range 240–300 mμ *B* part of globin absorption band in 290 mμ region at high absorbance showing tryptophan inflexion in adult carboxyhaemoglobin *C* as for *B* but foetal carboxyhaemoglobin at high absorbance showing resolved tryptophan fine structure band (100 % Hb F prepared by controlled alkali treatment of cord blood) (White & Beaven 1959)

(b) *Spectrographic methods*

These are based on the observation by Jope (1949) that the tryptophan fine structure band in the globin absorption band of haemoglobin (Fig 3) occurs at a shorter wavelength (289 8 mμ) in cord blood (about 80% Hb F) than in adult blood (291 0 mμ) For the location of the fine structure band which in Hb A is only an unresolved inflexion (Fig 3) Jope used a rather specialized but very convenient method devised by Holiday in which the photographic plate of a quartz spectrograph is moved by a logarithmic cam to give a relative overall exposure ratio of 1/100 The resulting spectrogram

* See above p 88

is particularly well suited for the detection and location of fine structure features which do not show up well on conventional plots of absorption spectra Jope s observation was extended by Beaven Hoch & Holiday (1951) to the analysis of Hb F/A mixtures and shown to be accurate to ± 5 – 10% over the range 10–90% Hb F but insensitive below the 10% level In this form the method has been applied to the estimation of Hb F in a wide variety of blood samples (White & Beaven 1954) It was used by Liquori (1951) to demonstrate the presence of Hb F in Cooley s anaemia bloods

A variant of the method applicable to commercial photo electric spectro photometers was devised by Rich (1952) who used the absorbance incre ment over the wavelength range 288 0–289 0 mμ to characterize the slope of absorption curve in the region of the tryptophan band after normaliza tion to a standard absorbance value at 290 0 mμ The method was used by Rich to estimate Hb F in Cooley s anaemia bloods Although it can be carried out on readily available equipment high experimental precision is required to obtain reliable results and the errors appear to be most serious with low proportions of Hb F (Beaven Ellis & White 1960)

(c) *Combined residue and spectrographic methods*

In order to extend the sensitivity of the spectrographic method below the 10% Hb F level Beaven Ellis & White (1956 1960) have applied it to alkali denaturation residues as obtained in the Singer method but with a reaction time of ¾ min and 2 5% HbO concentration By combining these two procedures Hb F may be estimated down to the 1% level and traces detected to about 0 5% In addition a correction for chemical damage to the haemoglobin in the residue may be applied in the form of a protein/haem ratio obtained by absorbance measurements at selected wavelengths This correction was introduced after it was found that Singer 1 min residues from normal adult blood had abnormal protein/haem ratios (2 or higher) due to loss of haem Residues from bloods containing more than 10% Hb F have protein/haem ratios much nearer unity and the correction rapidly becomes smaller as the level of Hb F rises above this limit The combined residue spectrographic method has been found to be of great value in studies of the survival of Hb F in early infancy and of its occurrence with other haemoglobin variants in the genetically determined haemoglobinopathies Apart from the high levels of Hb F encountered in Cooley s anaemia (thalassaemia major) such studies require a method suitable for Hb F levels in the range 1–20%

PHYSIOLOGICAL OCCURRENCE OF Hb F

(a) Infancy and childhood

Beaven Hoch & Holiday (1951) found no more than 10% of Hb F persisting 4 months after birth and it is difficult to detect any by the ordinary methods in older infants However Singer Chernoff & Singer (1951) and Jonxis & Visser (1956) thought that small percentages might be found for as long as 1½–2 years in the blood of healthy infants Using the

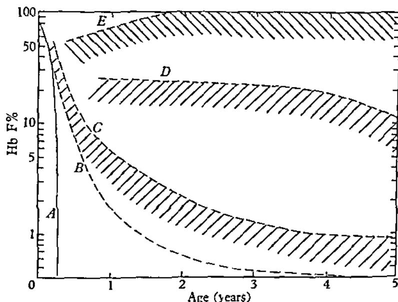


Fig 4 Foetal haemoglobin levels from birth to five years *A* curve corresponding to assumption of linear disappearance of Hb F (80% at birth) with the mean red cell life of days extended to below 1% survival *B* average Hb F levels in a series of child hospital patients with illnesses not involving abnormal haemoglobins *C* approximate upper level of Hb F in cases of thalassaemia minor and sickle cell trait *D* approximate upper level of Hb F in cases of sickle cell anaemia heterozygotes for two abnormal haemoglobins or thalassaemia and an abnormal haemoglobin (i e sickle-cell/Hb C disease sickle cell/thalassaemia) *E* approximate upper level at about 100% Hb F for cases of thalassaemia major

The Hb F values in the range below 1% are at the useful limit of the combined residue and spectrographic method and should be regarded only as indicating Hb F trace less than 1% Hb F values below 0 4% would be regarded as nil (White & Beaven 1959)

combined alkali denaturation and spectrographic method (Beaven Ellis & White 1956) we have not found more than traces (~1%) of Hb F after the first few years of life and the persistence of higher levels of Hb F in older children appears to be associated with hereditary haemoglobinopathies or occasionally with other severe blood disorders (Fig 4) While it is clear that cells containing Hb F continue to be formed after birth, this process is very limited in degree and finally ceases altogether in health

(b) Normal adult life

Despite much work it is still not clear whether a minute trace of Hb F may persist in normal adults Chernoff (1953) has claimed to demonstrate traces immunologically and Jonxis & Huisman (1956) believe the Singer residue is a mixture containing some Hb F and that this is supported by the amino acid composition (Huisman Jonxis & Dozy 1955) Beaven Ellis & White (1956) have found no spectrographic evidence for Hb F in residues from normal adult blood with a limit of sensitivity of 0 4–1 % in the original sample

PATHOLOGICAL SIGNIFICANCE OF Hb F

It has frequently been considered that Hb F may persist after birth in abnormal amounts or reappear in various pathological conditions The evidence is somewhat conflicting and although there is clear evidence for the post natal existence of Hb F in a number of conditions the mechanism of formation and teleological significance is by no means clear

Rubowitz (1933) suggested that with residence at high altitudes the low oxygen might stimulate formation of Hb F In our own work we have found no abnormal persistence in children with congenital cyanotic heart disease in long continued cyanosis due to drugs or in the myeloid hyper activity of myeloid metaplasia Despite pronounced marrow activity in hypochromic anaemia due to iron lack or blood loss Hb F appears to be absent

The resemblance of megaloblasts to primitive embryonic erythroblasts and the presence of abnormal macrocytes has suggested the presence of Hb F in pernicious anaemia (see Larsen 1951) though tests other than alkali denaturation did not confirm this (Iversen & Larsen 1956) By the sensitive combined method we have found traces (< 5 %) of Hb F in some untreated cases disappearing slowly with therapy In some cases of refractory anaemia with hyperplastic marrows small amounts have been detected although usually absent

The acquired haemolytic anaemias do not possess Hb F and it is usually absent in congenital spherocytic elliptocytic and atypical form though traces occasionally occur (Singer Chernoff & Singer 1951 White & Beaven 1954) Conditions with pronounced extra medullary haemopoiesis might be expected to form Hb F but it is usually absent or present only in small amounts in myelosclerosis and most leukaemias Occasionally however children with leukaemia form large amounts of Hb F (Beaven & White 1953)

The hereditary haemoglobinopathies

The hereditary haemoglobinopathies include quite a wide range of haemato logical disorders in which there is expression of the genes for thalassaemia sickling or for the formation of other 'abnormal haemoglobins It is in this group that abnormal persistence of Hb F in post natal life is particularly found

(*a*) *Thalassaemia* In the severely anaemic homozygous major form of thalassaemia large amounts of Hb F are of frequent and well established occurrence (Vecchio, 1946 Liquori 1951 Rich 1952) We have found a range of less than 20% to more than 90% in this condition The amount of Hb F is not particularly related to severity (Sturgeon Itano & Bergren, 1955*a*) and the markedly defective production of Hb A appears to be part of an inability of the hyperplastic bone marrow to form and deliver mature erythrocytes into the circulation (Sturgeon & Finch 1957)

A trace of Hb F may be found in adults with heterozygous thalassaemia minor (Singer Chernoff & Singer 1951) and we have found that about half these individuals have 1–4% of Hb F and that this is a useful diagnostic feature The relationship of Hb F to Hb A and the A_2 minor fraction in the various forms of thalassaemia has been discussed by Marinone & Bernasconi (1958)

Thalassaemia is very widespread geographically occurring widely in the Middle East and India as well as the Mediterranean area In the British Isles rare examples occur (Harvard Lehmann & Scott 1958) We have studied five families in which an hereditary hypochromic anaemia of this type has occurred and the finding of small quantities of Hb F in various members has been a valuable aid to diagnosis

(*b*) *Sickle cell disease* The sickling phenomenon is not easily elicited in the blood of young infants and as the Hb F declines during the first months of life Hb S appears in the cells and these can then be sickled (see Schneider & Haggard 1955) It is well known that in homozygous sickle cell anaemia although Hb S forms the bulk of the haemoglobin up to 20% of Hb F may accompany it The Hb F may exert some protective function since it does not enhance the gelation of reduced Hb S (Singer & Singer 1953) In the heterozygous sickle trait condition the Hb S is accompanied by an excess of Hb A and we have found only traces of Hb F present in about half the individuals examined

(*c*) *Other abnormal haemoglobins and mixed syndromes* Homozygous states for abnormal haemoglobins such as C and E may exhibit small amounts of Hb F but particularly notable amounts of up to 20–30% of the total may occur in the mixed syndromes due to the inheritance of the

thalassaemia gene and a second gene for Hb S C or E In these states the formation of Hb A appears suppressed to varying degrees the abnormal haemoglobin predominates but a varying proportion of Hb F co exists (see Itano Bergren & Sturgeon 1956 MacIver Went & Cruickshank 1958)

Though the question has been raised whether the Hb F found in pathological blood is identical with the normal foetal pigment (Perosa & Bini 1954) most workers agree that this is so (Itano 1957) Our own use of the simultaneous spectroscopic and alkali denaturation criteria support this conclusion

(*d*) *Genetic aspects of Hb F* The genetic pattern for the production of Hb F and its normal replacement by HB A is obscure The persistence of Hb F is of great interest in the many instances of operation of genes for the mendelian dominant abnormal haemoglobins most marked where homozygous much less so where heterozygous with the gene for Hb A production The marked influence of the gene for thalassaemia in continued Hb F production is also very evident when heterozygous with a further gene for an abnormal haemoglobin The possibility of double heterozygosity may be related to the great variability of Hb F and Hb A in such conditions and calls for careful familial as well as biochemical studies (Zuelzer 1957) The possibility that Hb F itself may persist into adult life as a genetic entity without associated haematological abnormality has been raised by Jacob & Raper (1958)

Recently important new information has been obtained on the structure of the globin of Hb F and its genetic significance *

THALASSAEMIA

The condition known as thalassaemia occurs commonly in members of the Mediterranean races The anaemia is characterized by considerable pleomorphism of the erythrocytes with thin hypochromic target cells with an increased osmotic resistance to saline haemolysis An inherited defect of haemoglobin synthesis has been suggested in thalassaemia (Damashek 1943 Wolman & Dickstein 1946) with inability to form normal adult haemoglobin but continued formation of a variable amount of foetal haemoglobin (Rich 1952) although the disease is probably not simply a haemoglobinopathy Considerable amounts of stainable iron occur in the reticulum cells of various organs apparently due to defective utilization for haemoglobin synthesis The erythroblasts may contain polysaccharide inclusions (Astaldi Rondanelli Bernardelli & Strosselli 1954)

* See below p 107

Crosby & Akeroyd (1951) have calculated that the rate of erythrocyte production by the marrow is not as great in thalassaemia as in some other haemolytic anaemias, and Sturgeon & Finch (1957) conclude that although the increased marrow turnover compares with other haemolytic anaemias there is a markedly decreased fabrication and delivery into the circulation of erythrocytes, which seems to be the cause of the anaemia rather than a defect in total haemoglobin synthesis

It has been known for some time that minor haematological abnormalities occur in the blood of both parents of children with severe thalassaemia and it is now well established that the severely affected cases are homozygous for the dominant variably expressed thalassaemia gene, whereas the carriers of the trait are heterozygous (Valentine & Neel 1944) Homo zygous thalassaemia major or Cooley s disease is usually manifest in early infancy and always in the first few years of life, death usually occurs in childhood, but survival to adult life may occur The heterozygous state of thalassaemia minor is very variable in its haematological constitutional and skeletal effects, and the Italian authors in particular distinguish moderately anaemic Mediterranean haematological syndrome or Rietti–Greppi–Micheli disease (Rietti 1925 Micheli, 1929 Greppi 1928) from thalassaemia minima or microcythaemia (Silverstroni & Bianco, 1943–4) in which the erythrocyte count is normal or raised, but the cells are small hypochromic and more resistant than normal to saline haemolysis

Although originally thought to be largely confined to Italians Greeks and other peoples of Mediterranean origin thalassaemia is now known to be considerably more widespread (Chernoff 1959) and to occur widely in the Middle and Far East including parts of India though not common in Tropical Africa There is considerable variability in the expression of thalassaemia apparently due to a combination of environmental and genetic modifiers An important group of mixed syndromes involve genes for thalassaemia and an abnormal haemoglobin An example of this is micro drepanocytic or thalassaemia/sickle cell disease (Silvestroni & Bianco 1952)

Reports appear in the literature of rather rare haemolytic anaemias occurring in individuals of pure Anglo Saxon or North and Central European stock and which are often of proven familial incidence and possessing some of the characteristic features of thalassaemia Electro phoresis of haemoglobin on starch slabs has shown that a small sub fraction of adult haemoglobin (Hb A) which is now designated A normally migrates more slowly than the main fraction at pH 8 5 and similarly to Hb E (Kunkel & Wallenius 1955) This fraction can also be separated well on starch gel electrophoresis (Gerald & Diamond 1956) or less clearly

on horizontal paper electrophoresis and is significantly raised above normal in most cases of thalassaemia minor and also in thalassaemia major as compared with the proportion of Hb A rather than the total haemoglobin (Kunkel Ceppellini Muller Eberhard & Wolf 1957 Marinone & Bernasconi 1958)

Many of the features of thalassaemia indicate a suppressed formation of Hb A with some compensatory degree of continued Hb F produced (Rich 1952) The nature of the defect in Hb A production is not known and it is also not certain whether the defect resides in the synthesis of ferroprotoporphyrin or of the globin Bannerman Grinstein & Moore (1959) have followed haemoglobin biosynthesis with labelled glycine or ^{59}Fe in immature thalassaemic blood cells *in vitro* and conclude that there is an overall quantitative impairment of haemoglobin biosynthesis not specifically connected with the globin but apparently due to a relatively slow rate of protoporphyrin synthesis or perhaps a block in the union of Fe^{++} with the latter Ingram & Stretton (1959) have recently suggested that a great slowing down in Hb A production would result from mutation in either of the genes required for the production of the α and β chains of the protein moiety of the normal pigment

THE HAEMOGLOBINOPATHIES

A large number of genetically determined variants of normal adult haemoglobin (Hb A) are now known showing various modifications of the globin structure and often involving very small departures from normal in that a single amino acid residue at a particular point in one of the polypeptide chains is altered * The first of these variants to be recognized and one of the most fully studied is sickle cell haemoglobin (Hb S)

Sickle cell disease and Hb S

Sickle cell trait and disease were formerly reported most commonly in the negro population of the United States of America This was due to lack of adequate medical study of the question in Tropical Africa since the American Negro is essentially of Central African origin and more recent studies reveal that sickling is of widespread though variable distribution in African as well as in various non African races (Lehmann 1954) Lehmann & Raper (1949) found 45 % of sickling among some African communities in Uganda and Edington (1953) recorded 18 % for the Accra Region of Ghana Allison (1954) considers that the trait may partially protect the bearer from the malarial parasites The incidence of sickling in

* See below p 105

Jamaica is up to 5 7% (Jelliffe, Stuart & Wills, 1954) In Great Britain the recent influx of Jamaicans has resulted in both sickle trait and various forms of sickle cell disease being encountered in hospital practice

The essential pathological condition which causes sickling of the erythro cytes is now known to be due to the presence within them of the abnormal haemoglobin S This type of haemoglobin is far less soluble than normal haemoglobin in the reduced state

Sickle cell disease is an inherited condition, and the presence or absence of the sickling phenomenon appears to be determined by a single gene The child who receives this gene from one parent and a normal gene from the other develops the sickle-cell trait (heterozygous state), but does not usually exhibit symptoms and does not become anaemic On the other hand the child who inherits the genes for sickling from both parents develops sickle cell anaemia (homozygous state) Occasionally sickle cell anaemia may be found in a child although the erythrocytes of only one of its parents sickle *in vitro* Neel (1952) suggested that the most likely explanation was that the apparently normal parent had contributed another gene which in com bination with a sickle cell gene produced an overt sickle cell anaemia

A brief historical survey of the recognition and nature of the disease is of interest In 1910 Herrick described many of the characteristic haemato *logical and clinical findings now referred to as sickle cell anaemia Emmel* (1917) suggested that the sickle cell phenomenon might be inherited It was later realized that sickling occurred in two distinct conditions (*a*) in a peculiar type of severe anaemia—sickle cell anaemia and (*b*) as a symptomless trait—the sickle cell trait (Cooley & Lee 1926 Neel 1949)

Taliafero & Huck (1923) again observed that the sickling phenomenon was inherited and suggested that the mode of inheritance was that of a men delian dominant Neel (1951) has postulated that the sickling phenomenon is due to a gene which in single doses produces the heterozygous trait in double doses the homozygous state with development of sickle cell anaemia

In 1949 Pauling Itano Singer and Wells demonstrated by electro phoresis that sickling was associated with an abnormal haemoglobin now called S In the trait S occurs together with excess of normal adult A haemoglobin whereas in the anaemia all the haemoglobin is S or some foetal haemoglobin accompanies it (Singer Chernoff & Singer 1951) The peculiar physical property of S haemoglobin which leads to distortion and sickling of the erythrocyte at low oxygen tension depends on the very low solubility of the reduced form (Perutz & Mitchison 1950) A small difference in a peptide group between normal and S *haemoglobins has* recently been found (Ingram 1956 1959) *

* See below p 105

In recent years it has been realized that genetically mixed forms of atypical sickle cell disease occur one of the patient s parents possessing sickle trait and the other some abnormal trait affecting haemoglobin or erythrocytes Such cases are less severe than homozygous sickle cell anaemia but present in a wide variety of clinical forms (Smith & Conley 1953 1954)

Other abnormal haemoglobins

A large number of types of haemoglobin have now been distinguished apart from normal haemoglobin (A) and foetal haemoglobin (F) Electro phoretic studies have distinguished S the sickling type and also C D E G H I etc These are genetically determined and in some cases may interact with the sickle cell gene to produce distinct anaemia The frequency of distribution of these haemoglobins varies greatly as does their significance in giving rise to overt haematological disorder Quantitatively haemo globins C and E are the most important after Hb S

Haemoglobin C occurs particularly in West African negroes with an incidence of up to 12% in parts of Ghana (Edington & Lehmann 1954) It is also found in American negroes and in Jamaica The heterozygous trait condition is of little immediate haematological importance the red cells are frequently of target form and contain a mixture of Hb A and Hb C The homozygous state may be associated with a mild haemolytic anaemia (Terry Motulsky & Rath 1954) and the cells contain Hb C with a minor proportion of Hb F

Haemoglobin D is much rarer but has an incidence of 3% in North West Indians and 1% in Gujeratis (Lehmann 1957) Haemoglobin E is of much more extensive distribution and is of very widespread occurrence in South East Asia In Thailand the incidence is 13 6% (Na Nakorn Minnich & Chernoff 1956)

Haemoglobins C D and E have characteristic electrophoretic mobilities the anodic mobilities on paper electrophoresis at pH 8 6 being progressively smaller than Hb A in the order A D E C (Plate 1) The mobility of Hb D resembles that of S but the solubility in the reduced state is normal The mobility of Hb E is very similar to that of the normal minor com ponent A_2 (Kunkel & Wallenius 1955 Kunkel Ceppellini Muller Eberhard & Wolf 1957)

A combination of these haemoglobins with sickle cell haemoglobin appears to explain many atypical sickle cell anaemias Several possible combinations are now recognized (Smith & Conley 1954 Itano Bergren & Sturgeon 1956)

(1) A combination of haemoglobins S and C in haemoglobin C sickle-

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* See below p 105

PLATE I



El ctrophore of some human haemoglobin variants The pap r strips are photographed directly after drying Cathode on l ft (White B aven & Ell 1956)

No 1 MD m cro-drepanocyt c d as
2 SC s ckl c ll—haemoglob n C d sea e
3 SD s ckl cell—haemoglob n D disease
4 DT D t ait
5 ST sickle cell trait
6 SCA s ckl c ll anaem a
7 A normal adult

cell disease (i e the subject has inherited one gene for haemoglobin S formation from one parent one for haemoglobin C from the other)

The clinical picture is largely that of sickle cell anaemia though milder and the disease follows a relatively more benign course There is a definite tendency to develop thrombotic lesions, due to impacted masses of sickled cells, in many parts of the body (Smith & Conley 1953 1954)

Smith & Conley (1954) found pregnancy to be more hazardous in women with haemoglobin C sickle cell disease than in sickle cell anaemia Edington (1955) has reported that among thirteen women whose blood sickled and who died suddenly in the last three months of pregnancy neither sickle trait nor homozygous anaemia was present in a number of the cases and at least one suffered from haemoglobin C sickle cell disease

(2) A combination of sickle cell haemoglobin and haemoglobin D (i e one gene for sickle cell haemoglobin and one gene for haemoglobin D) (Sturgeon Itano & Bergren 1955*b* White & Beaven 1954 Stewart & MacIver 1956)

(3) Microdrepanocytic disease, a combination of sickle cell and thalassaemia disease (i e one gene for sickle cell haemoglobin and one gene for thalassaemia or Mediterranean anaemia) This is a fairly severe condition encountered not infrequently in southern European and Middle Eastern countries bordering the Mediterranean as well as in American negroes (Silvestroni & Bianco 1952 Powell Rodarte & Neel, 1950 Humble Anderson White & Freeman 1954)

(4) Congenital spherocytosis—sickle cell disease (inheritance of genes for acholuric jaundice and sickling) (Smith & Conley 1954)

In Thailand both thalassaemia trait and haemoglobin E trait are of common occurrence so that in addition to homozygous children with thalassaemia major a moderately severe form of thalassaemia is seen in individuals heterozygous for both thalassaemia and Hb E In this latter condition Hb A formation is suppressed and the red cells contain Hb E as the major component, with up to 40% of Hb F

Haemoglobin H is a rather rare haemoglobin variant which possesses great instability and tendency to spontaneous denaturation (Rigas Koler & Osgood 1956 Gouttas Fessas Tsevrenis & Xefteri 1955) It gives rise to characteristic dye containing inclusions of denatured haemoglobin when the red cells containing it are incubated with brilliant cresyl blue in isotonic saline as for a reticulocyte preparation It also has characteristic electrophoretic properties moving ahead of Hb A on paper at pH 8 6 and at pH 6 4 the charge and direction of movement is opposite to that of Hb A (Plate 2A)

The parents of children with Hb H do not possess the abnormal haemo

globin but one of them usually has thalassaemia trait It is considered that the synthesis of Hb H is suppressed in the heterozygotes except in the simultaneous presence of the thalassaemia gene as well (Motulsky 1956 Vella 1957 Neel 1958) The blood picture resembles that of moderately severe thalassaemia with markedly hypochromic and pleomorphic red cells

A variant of normal Hb F Bart s or fast foetal haemoglobin (Ager & Lehmann 1958) has been detected in Hb H disease (Fessas & Mastrokalos 1959)

A rare example of an abnormal haemoglobin which causes a blood picture similar to thalassaemia trait is that found in Lepore trait (Gerald & Diamond 1958*a*) In the family studied by Gerald and Diamond one of the offspring of the mother with Lepore trait and father with thalassaemia trait suffered from severe thalassaemia with a great excess of Hb F accompanying Lepore haemoglobin in the red cells Both genetic abnormalities were apparently inherited simultaneously in this child

Haemoglobin abnormalities of acquired character

In three cases of erythro leukaemia in middle aged adults inclusion bodies have formed in the red cells on *in vitro* incubation with brilliant cresyl blue (Plate 2B) and have had features similar to the Hb H inclusions Haemo lysates from these cases also showed a small fast fraction on paper electro phoresis at pH 8 6 and abnormalities on starch gel agar gel and free boundary electrophoresis (White Ellis Coleman Beaven Gratzer Shooter & Skinner 1960) Although similar in some respects to Hb H this abnormal haemoglobin was probably distinct and it also appeared to exist as an acquired rather than a hereditary defect

Electrophoretically abnormal haemoglobin fractions may also occur in lead poisoning (Marder & Conley 1959)

TECHNIQUES FOR THE STUDY OF ABNORMAL HAEMOGLOBINS

Electrophoretic methods have been the most widely applied in the detection and discovery of abnormal haemoglobins The widespread application of filter paper electrophoresis and its convenience for rapidly examining large numbers of samples have been important factors

Filter paper electrophoresis

Electrophoresis is usually carried out on Whatman no 1 or the thick 3 mm paper the strips running horizontally The buffer most commonly employed is barbitone at pH 8 6 and *I* 0 05 Borate buffers may also be

Anode H A Origin

(i)

(ii)

(iii)

A

Anode H A

A Paper electrophoresis of haemoglobin in Hb H disease (i) and (ii) in barbitone buffer at pH 8·6 I 0·05 (iii) in phosphate buffer at pH 6·4 I 0·04 Photographed by direct transmission of violet light (i) and (iii) Hb H disease (22 % Hb H) (ii) Hb A



B Inclusion of abnormal haemoglobin in the brilliant cresyl blue preparation of red cells from a case of erythroleukaemia Fixed in Susa fluid and stained in aniline blue-orange G × 1280

Other methods of zone electrophoresis

(*a*) *Starch block electrophoresis* Kunkel & Wallenius (1955) applied their method of using a slab of starch grains covered with polythene sheeting to the electrophoresis in pH 8 6 barbitone buffer (*I*, 0 05 or 0 1) of red cell haemolysates Good separation of Hb A from haemoglobins S and E was obtained In addition the minor component of normal haemolysates now recognized as A_2 was clearly separated and found to possess the same behaviour as Hb E This minor component was increased in thalassaemia trait

Further work (Kunkel *et al* 1957 Marinone & Bernasconi 1958 Gerald & Diamond 1958) has shown that excellent separations of commonly encountered haemoglobins are obtained that sufficient quantities of haemoglobin can be applied for effective elution of the zones and that minor fractions such as A_2 can be estimated It has become apparent that the normal level of A_2 is about 2 5% of the total pigment and that a wide range of increased values is found in thalassaemia minor with a mean value about twice the normal However this increase may not be invariable in persons with other indications of the condition A small fast running A_3 fraction is also found in normal and other haemolysates and it has been suggested that this may be a degradation product of Hb A (Kunkel & Bearn 1957)

The technical aspects of the procedure are well discussed by Masri Josephson & Singer (1958)

(*b*) *Starch gel electrophoresis* Electrophoresis on starch gel (Smithies 1955) provides a method with great resolving power for the separation of proteins The application to human red cell haemolysates has been particularly concerned with minor components but the procedure has wide general application (Owen & Got 1957 Goldberg 1958) Tris (hydroxymethyl) aminomethane barate or barbitone buffers at pH values between 8 6 and 9 0 are used

(*c*) *Agar gel electrophoresis* Robinson Robson Harrison & Zuelzer (1957) applied electrophoresis on agar gel to the separation of haemoglobins in 0 05 M citrate buffer pH 6 2 Haemoglobins A S and C were separated in a manner analogous to that occurring on paper but Hb D differed from its behaviour on that medium in that it did not move like S and failed to separate from A Foetal haemoglobin also moved uniquely in a position ahead of Hb A

The method combines electrophoretic and chromatographic elements Although very sensitive for separating small amounts of Hb F other fractions in normal and other haemolysates move in a similar position and the separation is not unequivocal (Marder & Conley 1959)

used or phosphate at pH 6 4 and I, 0 04 The results may be examined directly, with greater sensitivity by transmission photography in violet light, or after staining with naphthalene black Quantitative assessments can be made by scanning the strips The technique has been frequently described (see White, Beaven & Ellis, 1956 Goldberg, 1957)

The relative mobilities of some of the main abnormal haemoglobins in descending order of migration, on paper electrophoresis at pH 8 6 are

$$H > J > K > A > F > G > S = D > E = A_2 > C$$

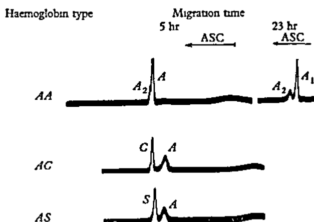


Fig 5 Electrophoretic analysis of normal adult sickle cell trait and haemoglobin *C* trait haemoglobins in phosphate buffer pH 6 4 *I* 0 04 Field strength 7 4 V/cm (From Shooter E M Skinner E R & White J C unpublished)

Moving boundary electrophoresis in the Tiselius apparatus

This powerful method for the identification of the abnormal haemoglobins has been applied by many workers Though much more time consuming and exacting than paper electrophoresis identification by absolute mobilities and the more certain resolution of mixtures of haemoglobins are possible

Numerous buffer systems are applicable and have considerable bearing on the results (Itano 1957) Phosphate buffer at pH 6 4 and I 0 04 (Fig 5) is particularly useful for the separation of Hb A and the minor A_2 fraction and for recognizing or resolving mixtures containing haemoglobins A, G S E and C (Shooter Skinner & White 1958) The relative order of mobilities is C > E > S > G > J with H migrating in the opposite direction In normal haemolysates A_2 migrates faster than Hb A Haemoglobin H is most conveniently recognized in cacodylate—NaCl buffer of pH 6 5 and I 0 10 (White *et al* 1960)

disease has both increased alkaline resistance and the ultra violet absorp tion characteristics of Hb F (Ager & Lehmann 1958) A haemoglobin encountered in Cyprus haemoglobin Cyprus I has increased alkaline resistance but in admixture with Hb A shows the normal adult type of tryptophane fine structure band (Gillespie *et al* 1959)

Haemoglobin M (Horlein & Weber 1948 Gerald Cook & Diamond 1957) possesses unique spectral characteristics in its derivatives which differentiate it readily from normal methaemoglobin Of the other abnormal haemoglobins only Hb A and Hb S are said to differ in certain fine respects in the visible region (McCord & Gadsden 1958)

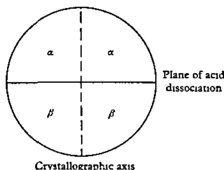


Fig 6

ADVANCES IN KNOWLEDGE OF THE STRUCTURE OF HAEMOGLOBINS

The haemoglobin molecule has an overall spheroidal form and possesses a dyad axis of symmetry which relates two identical half molecules (Perutz 1958 Cullis Dentzis & Perutz 1958) each containing an α and a β poly peptide chain (Shroeder 1959) Dissociation into half molecules occurs under acid conditions when the pH is lowered from 6 to 3 5 and is reflected in a fall in the sedimentation coefficient and increase in the translational diffusion constant on neutralization recombination occurs (Field & O Brien 1955) The dissociated half molecules however each con tain a pair of either α or β chains (Singer & Itano 1959*a b* Vinograd & Hutchinson 1959) A purely diagrammatic representation of the haemo globin molecule can be given as in Fig 6

Chemical differences between haemoglobins A S and C

Ingram (1959) has subjected heat denatured haemoglobin to tryptic hydrolysis followed by separation of the peptides by combined electro phoresis and chromatography Haemoglobins A S and C differ in respect

Ion exchange column chromatography

Boardman & Partridge (1955) separated mixtures of animal haemoglobin on the cation exchange resin 'amberlite IRC-50 This was extended to human haemoglobins by Morrison & Cook (1957) and Allen, Schroeder & Balog (1958) and both found up to 90% of normal haemoglobin in a main band about 10% of a faster minor component and variable results on the finding of slower minor components

Huisman & Prins (1955, 1957) have developed a method for elution of haemoglobin from columns by changes of buffer, and also a convenient practical method for developing a series of zones from haemoglobin mixtures in flat Lucite cuvettes Separations of haemoglobins A, S, C, E and F have been obtained Vella, Wells, Ager & Lehmann (1958) have extended the technique to haemoglobins H, F I (A J-K) G E (S D) L and C The method now forms part of the investigation and characterization of new abnormal haemoglobins

Solubility studies

The detailed studies of Jope and O Brien (1949) on the solubilities of the reduced HbO_2, HbCO and methaemoglobin derivatives of Hb A and Hb-F paved the way for the demonstration by Perutz & Mitchison (1950) that the significant physical abnormality in the sickling phenomenon is the very low solubility of reduced Hb S

Itano (1953) studied the solubility of reduced haemoglobins in phosphate buffer at pH 6 7 and high ionic strength The most noteworthy application in practice is in the distinction between Hb S and Hb D the latter possessing a normal solubility This finding in conjunction with the failure of the red cells to sickle and the failure of the haemoglobin to resolve from Hb A on agar gel electrophoresis, characterizes Hb D this haemoglobin itself forms a heterogeneous group however (Benzer, Ingram & Lehmann 1958)

The variable solute solubility method has been extensively applied to adult and foetal haemoglobins for the demonstration of heterogeneity (Roche Derrien Diacono & Roques 1953 Allison & Tombs 1957) A critical review of this and of other aspects of interpretation of the physicochemical properties of haemoglobins has recently been published by Beaven & Gratzer (1959)

Absorption spectra of haemoglobins

The main application of this technique has so far been in the recognition of Hb F in the presence of other abnormal haemoglobins * Bart s or fast foetal haemoglobin which may occur in association with Hb-H

* See above p 91

Haemoglobin H

This unstable haemoglobin possesses no α chains at all but consists of four normal β chains (Jones Schroeder Balog & Vinograd 1959)

Foetal haemoglobin

The same pair of α chains are present in Hb F as in Hb A but a pair of different γ chains replaces the β chains (Hunt 1959) The γ chains possess N terminal glycine residues (Schroeder & Matsuda 1958) The Bart s or fast variant of foetal haemoglobin appears to consist of γ chains only (Hunt & Lehmann 1959)

Hybridization

Itano and co workers have studied the recombination of acid dissociated half molecules of mixtures of two haemoglobins differing only in a β chain (e g A with S or C or C with S) During neutral recombination exchange of the half molecule common to the two forms occurs without the formation of a new molecular species (Itano & Singer 1958 Singer & Itano 1959*a b*) On the other hand recombination of two haemoglobins differing from each other in both chains apparently results in the formation of new molecular species which may be detected by electrophoretic methods An example is provided by a mixture of Hb S and Hb I (Itano & Robinson 1959*a*) This is a powerful technique for determining in which of the two polypeptide chains the alteration in an amino acid residue is occurring (Itano & Robinson 1959*b*)

Genetic aspects of haemoglobin structure

Studying a family in which combinations of haemoglobins G and S and thalassaemia occurred Schwartz Spaet Zuelzer Neel Robinson & Kaufman (1957) found that the genes for Hb S and Hb G and for Hb G and thalassaemia cannot be alleles Although the genes for haemoglobins S and C appear to be alleles (Ranney 1954) other family studies on microdrepanocytic disease (Silvestroni & Bianco 1952) indicated that the genes for Hb S and thalassaemia are not alleles and Schwartz *et al* concluded that several genetic loci are involved in haemoglobin production

The structural work on haemoglobins described above is beginning to yield some information on these points The differences between the abnormal haemoglobins depend upon relatively small changes in the polypeptide chains often involving a single site For example the mutational steps leading to formation of haemoglobins S and C from the normal allele for Hb A involve the synthesis of only the β polypeptide chain and

of a single peptide, and in particular with respect to one amino acid in this peptide

Hb A	Glutamic acid
Hb S	Valine
Hb C	Lysine

Hunt & Ingram (1959) have now separated the α and β *chains of these* haemoglobins by column chromatography on amberlite IRC-50 resin and applied the above procedure to each The differences between the *three haemoglobins are then found to reside in the* β chain, in peptide 4 which has the following sequences as determined by end group methods

Haemoglobin	Chain	Sequence
A	β^A	Val His Leuc Thr Pro *Glu* Glu Lys
S	β^S	Val His Leuc Thr Pro *Val* Glu Lys
C	β^C	Val His Leuc Thr Pro *Lys* Glu Lys

Rhinesmith Schroeder & Martin (1958) have found that the β chain which contains peptide 4 begins with the same tripeptide sequence, valyl histidyl leucyl as does peptide 4 This peptide is therefore probably at the N terminal end of the β chain

The general symbol for the structure of these three haemoglobins indicating the pair of α and pair of β chains per molecule is $\alpha_2 \beta$ Since the differences reside in the β chains Hb A may be written $\alpha^A \beta_2^A$ Hb S as $\alpha_2^A \beta_2^S$ and Hb C as $\alpha_2^A \beta^C$ (Rhinesmith Schroeder & Martin, 1958 Itano & Robinson, 1959*a b*)

Haemoglobin E

There appears to be an alteration in a single amino acid as compared with normal but though occurring in the β chain like S and C the site of alteration is different (Ingram 1959) It is likely that in Hb E the 26b peptide resembles that for Hb A except that a C-terminal lysine replaces glutamic acid (Hunt & Ingram 1959*a b*) This is the only difference so far detected and though it is similar to Hb C in that a lysyl residue is involved with similar charge effect the position is different

Haemoglobins G and D

In an example of haemoglobin D a glycyl residue has been found to replace a glutamyl in peptide 4, and it also differs from Hb S in containing only one valyl residue (Hill & Schwartz 1959) The abnormality is thus *adjacent to those in haemoglobins* S *and* C though differing in position Haemoglobin D samples from three different sources have been found to be heterogeneous in that each gave evidence of a different composition when studied by the *finger print technique* (*Benger Ingram* & *Lehmann* 1958) An example of an abnormality in the α chain has been provided by the rare haemoglobin I (Murayama & Ingram 1959)

ties and of methods for their recognition has aided diagnosis and knowledge of pathogenesis

The hereditary anaemias are assuming increasing importance and with other genetic disorders they present a great medical challenge of the day Originally a difficult field in which to make progress the study of human genetics now constitutes one of the most fertile fields of advance Bio chemical questions of protein synthesis and genetic problems of its control are being solved in a way which calls for the greatest admiration and the study of human haemoglobin can make proud claim to a major share of this

Grateful acknowledgement is made to Dr G H Beaven Medical Research Council Laboratories Hampstead and Dr E M Shooter University College London in respect of collaborative work discussed in this paper Financial support from the Colonial Medical Research Committee is also gratefully acknowledged

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then only replacement at a single glutamyl residue The α and β chains of haemoglobin may well be synthesized independently (see Allison 1959) and the actual haemoglobins appearing in the red cells in the haemoglobinopathies will be determined by the availability of these The normal change over from Hb F to Hb A production involves the continued production of α chains and replacement of γ by β chains (Hunt, 1959) Although the production of haemoglobin S, involving the β chain, is slow as compared with normal (Itano 1957) the production of Hb F is not affected in sickle cell disease This would be expected since haemoglobins F and S share a normal α chain and quite large amounts of Hb F may occur in sickle cell disease

Genetic relationship between haemoglobins S and E has been suggested (Aksoy & Lehmann 1957) and Hunt & Ingram (1959) find that in fact the single amino acid replacement in Hb E occurs in a peptide of the β chain though this peptide is different to the no 4 peptide in which the S and C replacements occur It may be expected that a good deal of light will be thrown on such relationships as the structure of the many haemoglobin variants is elucidated

Allison (1959) suggests that the genetic constitution of the normal individual for Hb A and Hb F synthesis may be expressed as $Hb\alpha^A/Hb\alpha^A$, $Hb\beta^A/Hb\beta^A$, $Hb\gamma^F/Hb\gamma^F$ In sickle cell trait the genetic constitution would be $Hb\alpha^A/Hb\alpha^A$ $Hb\beta^A/Hb\beta^S$, $Hb\gamma^F/Hb\gamma^F$, and so on for the other genetic variants in which the site of the polypeptide chain abnormality has been localized

The relationship of the chromosomal deoxyribonucleoprotein to the microsomal ribonucleic acid and the actual synthesis of the protein via its constituent polypeptide chains is still open to a good deal of uncertainty but it is evident that the synthesis of a particular protein is probably not under monolithic control of a single gene entity and is the result of the sum of the syntheses of its constituent polypeptide chains The genetic control of the amino acid sequences in these is a function of the purine pyrimidine base pair sequences in the chromosomal DNA and the single amino acid replacements which have been detected in the abnormal haemoglobins may well involve mutations in a very short sequence of these base pairs

CONCLUSION

A short survey has been given of the relevance of haemoglobin studies to the haematologist The various abnormal molecular species of haemoglobin as well as the various abnormal derivatives of the normal pigment are significant in relation to a number of anaemias The study of their proper

ties and of methods for their recognition has aided diagnosis and knowledge of pathogenesis

The hereditary anaemias are assuming increasing importance and with other genetic disorders they present a great medical challenge of the day Originally a difficult field in which to make progress the study of human genetics now constitutes one of the most fertile fields of advance Biochemical questions of protein synthesis and genetic problems of its control are being solved in a way which calls for the greatest admiration and the study of human haemoglobin can make proud claim to a major share of this

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CLINICAL AND CYTOLOGICAL RECOGNITION AND DIFFERENTIATION OF THE LEUKAEMIAS

F C J HAYHOE

Clinical examination can do no more than raise the suspicion that leukaemia may be present The suspicion may sometimes be strong enough to approach conviction but the establishment of a certain diagnosis is dependent upon finding characteristic changes in the blood or bone marrow Once the possibility of leukaemia has been envisaged on clinical grounds there is usually no difficulty in confirming or refuting the diagnosis by haematological examination Errors and delays in recognizing leukaemia arise chiefly from a failure to appreciate that the common modes of onset and the most typical presenting signs and symptoms are not the only ones and that a substantial minority of patients with leukaemia especially of the acute variety, have a clinical picture which conforms poorly with many textbook descriptions of the disease Misunderstandings about age incidence occasionally inhibit accurate diagnosis There is for example a widespread belief that acute leukaemia is predominantly a disease of childhood whereas it is the commonest form of leukaemia at all ages and probably has its maximum incidence among the elderly The differentiation of the varieties of acute leukaemia and the separation of the chronic leukaemias from certain non leukaemic lymphoproliferative and myeloproliferative states and from other leukaemoid reactions may be helped by clinical observations but is chiefly a problem to be solved by cytological and cytochemical studies In this paper I propose therefore to discuss in turn the acute leukaemias chronic granulocytic leukaemia chronic lymphocytic leukaemia and certain rare varieties of the disease dealing in each case first with the clinical aspects and then with cytological studies and drawing attention especially to diagnostic difficulties and how they may be overcome

ACUTE LEUKAEMIA

This is the most common form of leukaemia at all ages as may be seen from Fig 1 (constructed from the data of Gunz & Hough 1956) Its incidence is lowest in the 16–55 age group higher in children where the disease is nearly always acute and even higher in the elderly where all varieties of leukaemia show their maximum incidence Three chief subdivisions of

acute leukaemia are generally recognized myeloblastic, lymphoblastic and acute monocytic, this last including both pure monocytic and mixed myelo monocytic forms Clinically these varieties of acute leukaemia are very similar and the modes of onset and symptomatology may conveniently be dealt with in an inclusive manner emphasis being given where appro

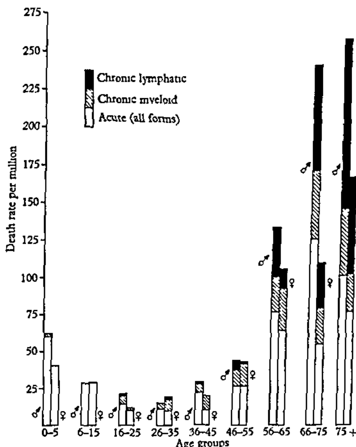


Fig 1 Incidence of leukaemia by age sex and type in New Zealand 1950–4 from the data of Gunz & Hough 1956 (reproduced with permission from Hayhoe F G J *Leukaemia—Research and Clinical Practice* J and A Churchill Ltd London 1960)

priate to features which are more common or more prominent in one variety than in others and which may therefore be of some help in classification It is of course important to classify cases of acute leukaemia as accurately as possible since the aetiology and pathogenesis of the different forms may not be identical and since therapeutic agents effective in one form may be less effective and even perhaps harmful in others

Modes of onset and symptomatology

The most typical form of onset and the one readily recognized by most clinicians as leukaemic is seen in children and in young adults In these patients the disease develops rapidly with fever headache weakness pallor dyspnoea on exertion and often scattered purpuric haemorrhages There may be skeletal pains and tenderness and sometimes joint swelling Gingivitis occurs not infrequently and may sometimes be severe Both liver and spleen are commonly palpable but neither of these organs is generally greatly enlarged and lymphatic glands too show only moderate increase Indeed in a proportion of these cases of acute leukaemia lymph adenopathy and hepato splenomegaly are totally absent or at least not detectable by palpation Since the abnormal leucocytic state renders the patient unduly susceptible to intercurrent infections it is not surprising that respiratory or urinary systems may be involved in some secondary infective process The initial pattern of symptomatology seen in any individual patient is dictated partly by the extent and location of leukaemic infiltrates and deposits and even more conspicuously by the secondary phenomena of thrombocytopenic bleeding infection and anaemia Never theless the clinical picture of acute leukaemia in young persons is suf ficiently characteristic for it rarely to remain undiagnosed for long At initial clinical examination however an incorrect diagnosis is made in a surprising proportion of patients In a survey of the records of a children s hospital in Germany covering the period between the years 1944 and 1955 Oehme (1957) found that only ten of ninety eight cases of acute leukaemia had been correctly diagnosed by the admitting physician Among the erroneous diagnoses were thirteen of haemorrhagic diathesis thirteen of contagious diseases including infectious mononucleosis influenza menin gitis poliomyelitis mumps scarlet fever and other forms of sepsis eleven of primary anaemia ten of rheumatism and endocarditis six of gastro intestinal diseases four of tuberculosis three of Hodgkin s disease three of panmyelopathy three of osteomyelitis and one each of bronchopneumonia glossitis buccal cellulitis and nephritis This assembly of incorrect diagnoses does something to illustrate the difficulties of early clinical recognition of acute leukaemia and suggests a number of pitfalls to be avoided Some of the less common modes of onset of the disease which have occasionally proved misleading may be listed as follows

(1) *Onset resembling that of an acute infection* When fever headache weakness and muscular pains are prominent and anaemia and purpura inconspicuous the onset may certainly resemble that of many severe infections especially in childhood and since there may indeed be some

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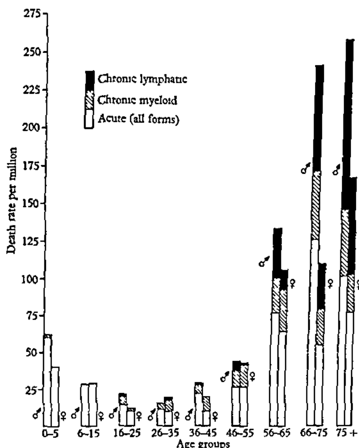


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pain and swelling of the larger joints accompanied by anaemia perhaps slight enlargement of the spleen but with no other clear pointer to leukaemia The literature of twenty five years ago includes descriptions of cases initially diagnosed as subacute rheumatism and treated with salicylates for several weeks before the true diagnosis of leukaemia became obvious The same sequence of events is certainly not unknown today yet the mistake could readily be avoided if at least one full blood examination were carried out on every child with suspected acute rheumatism The initial predominance of bone and joint symptoms is seen especially in children perhaps because of the lability of the growing skeletal system but occasion ally backache with perhaps the collapse of a vertebral body may provide the first sign of acute leukaemia in an adult

(4) *Onset with predominant glandular enlargement* Although as we have seen already lymph gland enlargement is not commonly great in acute leukaemia occasionally it may be considerable and may provide the first indication of disease Generalized lymphadenopathy of substantial degree is quite uncommon in myeloblastic and monocytic forms of leukaemia although it is occasionally observed in them In the lymphoblastic variety of the disease however while lymph glands are commonly a little enlarged they may sometimes show a very substantial increase perhaps localized to a single site and massive enlargement of lymph nodes in the mediastinum has occasionally given rise to acute respiratory or mediastinal obstruction as the initial leukaemic symptom Differential diagnosis from lympho sarcoma reticulum cell sarcoma Hodgkin s disease or even carcinoma of the lung or some other solid tumour may be impossible on clinical grounds alone Examination of the blood and the sternal marrow are such simple procedures that it is surprising to find patients with this pattern of initial symptomatology sometimes having lymph gland biopsies bronchoscopies or other elaborate investigations performed before blood count and marrow examination have been done

(5) *Onset with predominant skin lesions* Purpuric skin rashes occur almost invariably at some stage of the disease in every case of acute leukaemia but more specific dermatological involvement by leukaemic cellular infiltrates is uncommon in lymphoblastic and myeloblastic leukaemias and only a little more frequent in the monocytic form of the disease The nature of the skin rash found is extremely variable including maculo papular eruptions separated nodules plaques generalized pustular rashes and even exfoliative dermatitis In nearly all cases however there is at least some haemorrhagic element to the rash and even in the absence of other signs of leukaemia this phenomenon should provide a pointer to the possible diagnosis and lead to an examination of the blood

element of secondary infection of the respiratory or urinary system or elsewhere present, the primary disease may easily be overlooked at this stage

(2) *Onset with predominantly oral symptoms and signs* Bleeding from the gum margins and petechiae in the buccal mucosae often develop very early and appear to be equally common in all varieties of acute leukaemia Swelling and ulceration of the gums sometimes spreading to the soft palate and pharynx may also occur in any form of acute leukaemia but gross examples of oral sepsis and gingival hyperplasia are certainly most common in the monocytic variety where the teeth may be almost sub merged by the gum swelling When mouth involvement of this kind is found together with a variety of other signs of acute leukaemia it provides a valuable pointer to the diagnosis but when oral lesions occur in a patient who appears otherwise healthy the possibility of a systemic disease may not even be considered This is more often true perhaps when the oral involvement is not extreme with gross hypertrophy of the gums but when less striking mouth lesions are present, due perhaps to bacterial or fungal invasion Initial diagnoses of primary oral sepsis Vincent s angina or some condition of this kind may often then be made It would be unreason able to expect a full blood count to be carried out on every patient with a sore throat or other manifestation of oral or pharyngeal infection but it is certainly a wise plan to have such a blood examination performed on any patient with oral sepsis whose condition does not rapidly respond to conventional treatment more especially if gum hyperplasia or haemorrhagic signs are present

(3) *Onset with symptoms referring principally to the bones and joints* Aching pains in the back or in the long bones sometimes localized to a single site may be very severe and may occasionally be present as the first symptoms of acute leukaemia X ray changes in the bones can nearly always be demonstrated in patients with such complaints but the radio logical changes do not often show characteristic features and confusion with other diseases may still be present Zones of increased translucency at the metaphyses of long bones are similar to those found in many other childhood disorders while the more striking changes of erosion and infiltration with subperiosteal new bone formation which are occasionally seen in leukaemia may sometimes resemble the appearances of osteo myelitis or even osteogenic sarcoma Any patient with continued and severe bone pains especially in childhood should certainly have a blood count performed Involvement of joints in acute leukaemia is even more common and children especially may present with a clinical picture closely resembling that of acute or subacute rheumatism or Still s disease having

cells and the severe thrombocytopenia which is so conspicuous a feature of the disease When such a picture is present there is no difficulty in recognizing the disease The problems arise when the total leucocyte count in the peripheral blood is within or below normal limits as is the case at initial haematological examination in perhaps 25 or 30% of cases of acute leukaemia In most of these the difficulty is still a superficial one since al though the total leucocyte count is not raised the majority of the circulating leucocytes are primitive cells This picture may reasonably be called *sub leukaemic* to distinguish it from the genuinely *aleukaemic* picture in which no primitive cells appear in the peripheral blood When leukaemia is clinically suspected and the blood count shows anaemia and thrombocytopenia without diagnostic changes in the leucocytes bone marrow examination becomes imperative

Bone marrow aspirates in acute leukaemia are nearly always highly cellular and compo ed chiefly of nucleolated primitive cells This is true whether the peripheral blood is fully leukaemic subleukaemic or even aleukaemic Occasionally a poorly cellular specimen may be obtained perhaps because of irregular or patchy involvement of the bone marrow or because the cells at the site of puncture were so densely packed as to be difficult to aspirate through the sternal puncture needle Even when this is so the material obtained is usually composed predominantly of blast cells and enables a diagnosis to be made A second puncture at a different site may yield a more cellular and characteristic specimen Only rarely during certain aplastic phases of acute leukaemia may both blood and marrow be so poorly cellular as regards leucocyte precursors that differential diagnosis from aplastic anaemia or agranulocytosis may be temporarily impossible The occurrence of such aplastic phases preceding the development of frank leukaemia arising during the course of the disease and reverting once more to a leukaemic picture or forming the final episode of the illness has now been well established (Hayhoe 1957) and every patient with the findings of aplasia with pancytopenia should be considered as a possible example of leukaemia in an aplastic phase Nevertheless the marrow appearances in acute leukaemia at initial examination are clearly diagnostic in the vast majority of cases

Cytological differentiation of types of acute leukaemia

While more mature forms of leucocytes are easily distinguished from one another their immature precursors myeloblasts lymphoblasts and mono blasts have sufficient general similarities to make their recognition a matter often of considerable difficulty When fair numbers of promyelo cytes or monocytes are to be seen the accompanying blast cells may reason

(6) *Onset with other system localization* Initial predominance of symptoms and signs in the central nervous system the eyes, the ears, the alimentary tract the genito urinary system or elsewhere unaccompanied by general signs of leukaemia is sufficiently uncommon not to require any detailed consideration Provided the physician is aware that acute leukaemia must be considered whenever lesions possibly due to localized infection haemorrhage or infiltration are present and the causative process uncertain, the necessary blood or marrow examinations can confirm or deny the possibility of leukaemia without difficulty or delay

(7) *Insidious onset* In the majority of middle aged and elderly patients and also in some younger ones the abrupt onset classically described for acute leukaemia is not seen These patients show slowly progressive symptoms of anorexia lassitude exertional dyspnoea and the like and the symptoms have often been present for many months before medical advice is sought Physical signs at the first examination may like the clinical history, give little resemblance to the picture of acute leukaemia as commonly described in children and young adults and there may be little to find other than signs of anaemia or of conditions such as cardiac failure which may be secondary to anaemia In this group of patients diagnoses of aplastic or pernicious anaemia carcinomatosis chronic bronchitis cardiac failure and so on have often been made before investigation of the blood has revealed the true diagnosis It is perhaps noteworthy that the examples of *pre leukaemic states* described by Block Jacobson & Bethard (1953) and later by other authors have usually been observed in patients over the age of forty in whom apparently non leukaemic blood disorders had been present for periods of several or many months before frankly leukaemic changes developed In these patients with pre leukaemic states the clinical signs were generally those that one might expect in the presence of peripheral cytopenias with anaemia infections resulting from granulopenia and with haemorrhages secondary to thrombocytopenia The border line between such pancytopenic states and acute leukaemia in elderly patients is a nebulous one since even in clearly leukaemic elderly patients both peripheral blood and bone marrow may be poorly cellular though with a vast predominance of leukaemic blast cells

The blood and marrow picture in acute leukaemia

I do not propose to discuss in detail the blood and marrow pictures typical of acute leukaemia The general pattern of high leucocyte count in the peripheral blood ranging between 20 000 and 50 000 white cells per mm^3 the great majority of them being primitive blast cells is sufficiently familiar Equally familiar are the anaemia with either normocytic or macrocytic red

PLATE I



A Myeloblastic leukaemia—May Grunwald-Giemsa stain (MGG) B Lymphoblastic leukaemia—MGG C Acute monocytic leukaemia—MGG D Myeloblastic leukaemia—Periodic acid Schiff reaction (PAS) E Lymphoblastic leukaemia—PAS F Acute monocytic leukaemia—PAS

(Figs A F reproduced with permission in half tone from colour plates in Hayhoe F G J *Leukaemia—Research and Clinical Practice* J and A Churchill Ltd London 1960)

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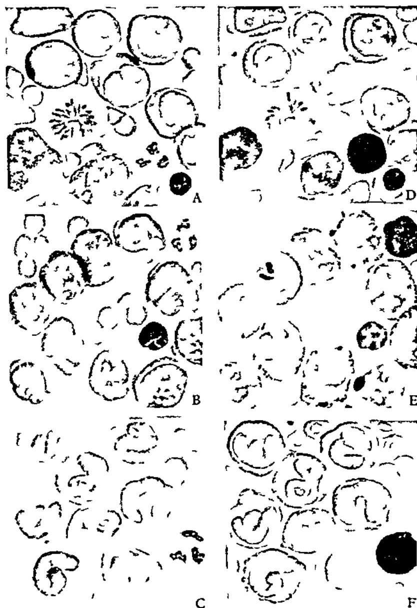
ably be taken to be myeloblasts or monoblasts respectively but when 80 or 90% of the white cells in blood and marrow smears are of uniform immature type with very few cells of an intermediate stage in development recognition is less easily achieved Many attempts have been made to establish criteria by which these primitive cells can be classified with certainty Let me say at once that no simple method has proved con sistently successful Probably a combination of cytochemical stains offers the nearest approach to definition In addition to orthodox Romanowsky and peroxidase staining we currently employ the Periodic acid Schiff (PAS) reaction for glycogen and related mucopolysaccharides Sudan black B staining for intracellular lipids, the Feulgen reaction for DNA and an alkaline phosphatase reaction (Hayhoe & Quaglino 1958) Sufficient studies have now been carried out on over a hundred cases of acute leukaemia to enable us to recognize, at least tentatively certain patterns of reaction generally characteristic of the three major varieties of acute leukaemia (Hayhoe 1959 Quaglino 1959) Typical appearances are illustrated in Plates 1 and 2 Apart from the differentiating features visible in Romanowsky and peroxidase preparations the findings of most help are those with the PAS and alkaline phosphatase reactions Myeloblasts are generally PAS negative whereas lymphoblasts are often strongly positive and monocyte precursors show a variable but usually fine granular posi tivity In myeloblastic leukaemia the mature polymorphs have very little alkaline phosphatase activity while in lymphoblastic and acute monocytic leukaemia they are usually rich in this enzyme

Phase contrast microscopy may also be helpful, when it is available since the mitochondria appear to be more scattered about the cytoplasm in myeloblasts than in lymphoblasts where they are clumped together at one side of the nucleus In monocytic leukaemia the cells usually show con siderable motility

Despite all these methods one must still admit that an occasional case fails to conform to the criteria but cases difficult to classify are now quite unusual whereas a year or two ago they were commonplace

CHRONIC GRANULOCYTIC LEUKAEMIA

This disease is rare in childhood but becomes more common with increasing age It has about equal frequency in men and women (Fig 1) The general pattern of onset is highly insidious and there is reason to believe that most patients have had the disease for as long as eighteen months or two years before seeking medical advice The commonest initial symptoms are weakness abdominal pain or consciousness of an abdominal tumour loss



A. Myeloblastic leukaemia—May-Grunwald Giesma stain (MGG) B Lymphoblastic leukaemia—MGG C Acute monocytic leukaemia—MGG D Myeloblastic leukaemia—Periodic acid Schiff reaction (PAS) E Lymphoblastic leukaemia—PAS F Acute monocytic leukaemia—PAS

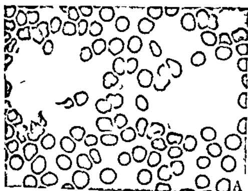
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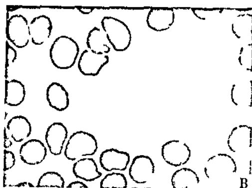
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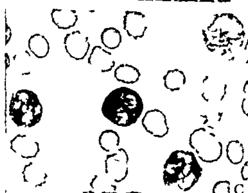


A *Myeloblastic leukaemia—Alkaline phosphatase reaction* B *Lymphoblastic leukaemia*—Alkaline phosphatase reaction C Acute monocytic leukaemia—Alkaline phosphatase reaction D Myeloblastic leukaemia—Sudan black B stain E Lymphoblastic leukaemia—Sudan black B stain F Acute monocytic leukaemia—Sudan black B stain

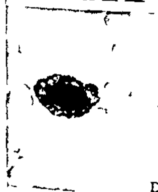
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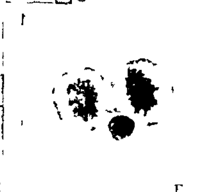














A Chronic granulocytic leukaemia—Alkaline phosphatase (low power) B Chronic granulocytic leukaemia—Alkaline phosphatase (high power) C Leucocytes in polycythaemia vera—Alkaline phosphatase D Disseminated eosinophilic cell Letterer–Siwe disease E A tissue mast cell F Erythraemic myelosis—dividing erythroblast (MGG stain) G The same field as in Fig F consecutively studied by the PAS reaction

of weight and breathlessness on exertion Examination reveals anaemia and considerable splenomegaly in the majority of cases Less often but more commonly than is generally realized the first sign of chronic granulocytic leukaemia may be the development of a haemorrhagic state Occasionally the initial complaint may be of poorly localized bone pains menstrual irregularities herniation or uterine prolapse secondary to splenomegaly skin rashes or pruritus or even rarely localized enlargement of lymph glands In nearly every case the spleen is enlarged usually grossly and since a blood count is always called for in the presence of splenic enlargement the diagnosis becomes clear without delay

Obviously there may be difficulty in distinguishing clinically between chronic granulocytic leukaemia and other causes of splenomegaly and anaemia but there is very little to be gained from an elaborate clinical comparison between these potentially confusing states since in any of them a blood count would certainly be performed which would either establish the diagnosis clearly or make it a matter for cytological rather than clinical differentiation

The blood and marrow picture in chronic granulocytic leukaemia

In the vast majority of cases the peripheral blood findings are quite characteristic from the time of first examination There are great numbers of circulating granulocytes or granulocyte precursors with neutrophil polymorphs metamyelocytes and myelocytes predominating but with up to 10% or so of promyelocytes and myeloblasts and commonly a conspicuous increase in the numbers of basophil and eosinophil leucocytes Total leucocyte counts are usually between 100 000 and 400 000 per mm^3 and counts below 50 000 per mm^3 at initial examination are uncommon A sharp increase in monocytes is sometimes seen Platelets are usually numerous at first although thrombocytopenia frequently develops later in the course of the disease A normocytic normochromic anaemia is usual and when the anaemia is severe there may be occasional normoblasts in the blood and reticulocytes may rise to 5 or 10%

The bone marrow is very actively cellular with increased myeloid erythroid ratio and the granulocyte pattern resembles that in the peripheral blood Islands of erythroblasts are to be found and probably the total erythropoietic activity is at least as great as in a normal subject Megakaryocytes are often conspicuous and may be obviously increased in numbers in the early stages of disease when the peripheral platelet count is high

I do not propose to analyse the relationship between chronic granulocytic leukaemia and other myeloproliferative states such as polycythaemia vera myelofibrosis and megakaryocytic myelosis since this forms the subject of

another lecture As for granulocytic leukaemoid reactions to infection or disseminated malignancy and the occasional high leucocytosis accompany ing haemolytic reactions or following haemorrhage, these are very seldom so like leukaemia as to cause much difficulty When difficulty does arise either with leukaemoid reactions or non leukaemic myeloproliferative states the leucocyte alkaline phosphatase level provides a remarkably reliable guide in differentiation since the neutrophil polymorphs in granulocytic leukaemia are almost devoid of the enzyme while those in both the other groups of disease are more than normally rich in it (Plate 3 A–C)

CHRONIC LYMPHOCYTIC LEUKAEMIA

The age incidence can be seen from Fig 1 The disease is rare before middle age but becomes increasingly common with advancing years The onset is even more insidious than that of chronic granulocytic leukaemia *and the initial disease picture is more varied The first complaint is most* commonly of enlarged lymph glands especially in the neck but other primary symptoms include lassitude loss of weight exertional dyspnoea abdominal pain awareness of a splenic tumour purpura specific skin rashes anginal pain and ankle oedema Intercurrent infections to which patients with chronic lymphocytic leukaemia are unduly prone sometimes bring the existence of the disease to light In many patients the blood condition is discovered unexpectedly during the investigation of an apparently unrelated condition and at this stage there may be few or no abnormal physical signs attributable to leukaemia Nevertheless at least 70% of patients with chronic lymphocytic leukaemia have generalized moderate enlargement of lymph glands at the time of first clinical examina tion and there is usually palpable but not gross hepato splenomegaly as well Perhaps 10 or 20% of patients show a picture like that of chronic granulocytic leukaemia with gross splenomegaly and only slight or absent lymphadenopathy A further small group show localized lymph gland enlargement clinically resembling Hodgkin s disease or lymphosarcoma Still more rarely purpura or skin infiltration may precede the development of other physical signs of disease as in leukaemic erythrodermia It is enough to be aware of these possibilities and to carry out examination of the blood for the true diagnosis to be made in nearly every case

The blood and marrow picture in chronic lymphocytic leukaemia

Appearances are usually quite characteristic The peripheral blood shows from *20 000 to 300 000 white cells per* mm^3 *predominantly mature* lymphocytes often with many smear cells Counts below 10 000 per mm^3

are not uncommon occurring in perhaps 10% of patients but even here lymphocytes greatly preponderate Anaemia is not usually striking initially but often develops later in the disease and may be haemolytic in character with sensitized red cells reticulocytosis and bilirubinaemia Platelets are generally normal at first though thrombocytopenia may occur in the terminal stages

The bone marrow shows varying degrees of infiltration with lymphocytes which usually make up from 30 to 80% of the marrow leucocyte population A parallel erythroblastic proliferation may be present if there is haemolytic anaemia

When marked lymphocytosis in the blood and marrow is found there is rarely any diagnostic difficulty Peripheral lymphocyte counts exceeding 10 000 or even 20 000 may be encountered in glandular fever pertussis and infectious lymphocytosis but all these diseases are rare in the middle aged and elderly Glandular fever may be separated also by the characteristic cytology of its lymphomononuclear cells and by the Paul–Bunnell reaction pertussis by its clinical picture and infectious lymphocytosis by its onset with fever and gastro intestinal infection and its benign course with rapid resolution

When lymphadenopathy and perhaps hepatosplenomegaly exist without convincing lymphocytic changes in blood or bone marrow lymph gland biopsy is necessary to establish a diagnosis If a pattern of lymphocytic hyperplasia is found without obvious malignant invasion of the capsule or mitotic overactivity the label lymphocytic lymphoma without leukaemia is sometimes applied In such cases clear changes of leukaemia may be expected to develop in the blood and marrow in the course of time Differentiation between these primarily glandular forms of chronic lymphocytic leukaemia and lymphocytic lymphosarcoma may be impossible and indeed there is no sharp distinction between the two diseases which share a common ill defined border line both clinically and pathologically

UNUSUAL FORMS OF LEUKAEMIA

I make only a brief reference to chloroma eosinophilic basophilic and mast cell leukaemias to plasma cell leukaemia and to erythraemic myelosis

Chloroma is a variety of acute myeloblastic or monocytic leukaemia in which tumour masses greenish in colour are found chiefly in relation to the periosteum and bones of the skull The clinical picture is like that of acute leukaemia in children or young adults with additional symptoms and signs due to rapidly growing tumours often in the orbit when blindness proptosis and oculomotor paralyses may develop Chloromatous

deposits may occur at many other sites elsewhere in the skeleton or in the lymph glands or viscera The blood and marrow findings are those of acute myeloblastic or myelomonocytic leukaemia

Eosinophilic leukaemia is an entity of very doubtful existence All recorded cases in which data are adequate appear to be examples of leukaemoid reactions transitory phases of eosinophilia during acute or chronic granulocytic leukaemias or of disseminated eosinophilic collagen disease (Plate 3 D)

Basophilic leukaemia is an equally doubtful entity The basophil count may occasionally be very high in chronic granulocytic leukaemia but this seems to be no more than an exaggeration of the customary basophilia present in this disease

Tissue mast cell leukaemia (Plate 3 E) has been recognized only recently Two cases have hitherto been reported They are of interest in that they provide a malignant extreme to a range of mast cell disorders which includes urticaria pigmentosa and various degrees of systemic mastocytosis

Plasma cell leukaemia is no more than a rapidly disseminating form of multiple myelomatosis having the usual hyperglobulinaemia and sometimes Bence Jones proteinuria There is no justification for regarding it as a separate disease

Erythraemic myelosis This malignant proliferative disease of erythroblastic tissue may occur without obvious parallel acute leukaemia but erythroleukaemic states intermediate between acute myeloblastic or myelomonocytic leukaemia and pure erythraemic myelosis are rather more common and indeed, pure erythraemic forms usually terminate with a transformation to apparent acute leukaemia Acute erythraemic myelosis clinically resembles acute leukaemia with rapid onset severe anaemia fever and a short malignant course Splenomegaly is perhaps more conspicuous and the liver is usually palpable The peripheral blood shows remarkable erythroblastosis proerythroblasts and basophil erythroblasts predominating with counts sometimes as high as several hundred thousand per mm^3 A chronic form of the disease occurs with slower clinical course and with later normoblasts predominating in blood and marrow When the peripheral erythroblastosis is minimal the disease is difficult to differentiate from refractory normoblastic or megaloblastic anaemias and it is possible that the two conditions are identical

Cytochemically the erythroblasts in erythraemic myelosis show free iron granules and are often conspicuously PAS positive with diffuse cytoplasmic tinting and coarse granularity (Plate 3 F and G) Strong PAS positivity in the erythroblasts occurs in severe iron deficiency anaemia and in thalassaemia but if these conditions can be excluded the presence of a strong

PAS reaction in many erythroblasts is extremely suggestive of erythraemic myelosis (Quaglino & Hayhoe 1960) The fact that refractory normoblastic or megaloblastic anaemias often show iron staining and PAS positivity somewhat resembling that of obvious cases of erythraemic myelosis is a further pointer to the possible identity of the two conditions

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THE MYELOPROLIFERATIVE SYNDROMES

G WETHERLEY-MEIN

During the last ten years the term myeloproliferative disorder has been increasingly used as a generic name for a large group of proliferative processes which most commonly, involve *lymph nodes bone marrow and* spleen and which includes the leukaemias the lymphomas myelofibrosis polycythaemia and similar conditions

The impetus which the early purely morphological classifications gave to the study of these conditions was considerable and should not be underrated, but the complex terminology which they generated—dictyocytic reticulum cell sarcoma, agnogenic myeloid metaplasia histiocytic medullary reticulosis, and so forth—became cumbrous confusing and impracticable The clinical presentation and the haematological and histological patterns which may be observed in these states are so varied that precise classification of the individual patient may be impossible, and the concept that all these conditions are intimately related members of a single group—the myeloproliferative disorders—has an attractive simplicity which has been largely responsible for its widespread acceptance (Custer & Bernhard 1948 Willis 1948) This concept which stresses a community of histogenesis and disregards dissimilarity of histology has by its simplicity enlarged our understanding of these processes and has of course obvious advantages in terms of diagnosis prognosis and management Simplifications particularly when related to biological processes are almost invariably over simplifications and may by common usage come to be regarded as unquestionable facts Therefore before discussing some specific syndromes in detail I should like to re examine the hypothesis from which this concept *of a closely related group of myeloproliferative disorders* has been developed

This hypothesis implies that a considerable number of conditions including the leukaemias and the lymphomas are expressions of an abnormal and often excessive proliferation of cells of primitive mesenchymal origin (Pullinger 1932 Ross 1933 Custer & Bernhard 1948 Willis 1948) Maximow (1927) considered that in the adult numbers of these cells persisted in such sites as spleen bone marrow lymph nodes and were in particular widely distributed in the perivascular connective *tissue These observations* which were based on studies of inflammatory processes and tissue cultures showed that these cells possessed the same capacity for manifold differentiation as embryonic mesenchyme that is

given an appropriate stimulus they could differentiate to form red cells granulocytes lymphocytes monocytes reticulum cells fibroblasts bone blood vessels etc The anatomical distribution and the biological possibilities of mesenchymal tissue (summarized in Table 1) suggest that stimuli which lead to abnormal proliferation of this system of mesenchymal cells may produce a wide variety of clinical and histological patterns The hypothesis gains considerable support from the fact that the majority of the possible permutations and combinations have in fact been observed

Table 1

Primitive mesenchymal cell

Distribution	Potential differentiation to
Marrow	Granulocyte
Lymph nodes	Lymphocyte
Spleen	Platelet
L er	Plasma cell
Perivascular connective	Monocyte
tissue n Gut	Histiocyte
Kidney	Reticulum cell
Dermis	Fibrocyte
Lung etc	Blood vessel
	Bone
	Cartilage etc

Simple uncomplicated examples of such proliferations are the classical chronic myelocytic leukaemia involving bone marrow and spleen pre dominantly lymphosarcoma involving predominantly lymph nodes and spleen and reticulum cell sarcoma involving predominantly again the lymph nodes and the spleen

Certain uncommon presentations also emphasize the widespread distribu tion of these cells and their capacity for differentiating in specific directions in unusual sites Chronic myelocytic leukaemia may for example present predominantly with massive enlargement of superficial lymph nodes and similarly (as shown in Plate 1A 1B) lymphosarcoma and leukaemia may initially and predominantly involve such sites as the gut or the dermis

Perhaps the most convincing evidence in support of this concept that the myeloproliferative syndromes are closely related manifestations of mesen chymal cell proliferation are combined proliferations of more than one cell type in the same patient or the transition from one type of proliferation to another in the same patient In Fig 1 for example is shown the extremely common combination of platelet proliferation and granulocytic prolifera tion found in many cases of chronic myelocytic leukaemia In this par ticular patient whose platelets were of the order of 1–2 million when she presented the initial symptoms were related to the excessive bleeding

which is often characteristic of thrombocythaemic states The close relationship between platelet and granulocyte proliferation is further emphasized in this patient by the parallel fluctuations of both cell types which so characteristically followed administration or withdrawal of busulphan Disturbance of this pattern with a rising white count and falling platelet count is an invariable indication, *in our experience* not of resistance to the drug but of impending transition to the myeloblastic phase We have now treated about 25 patients solely with busulphan and death when it has occurred has been the result of myeloblastic transition

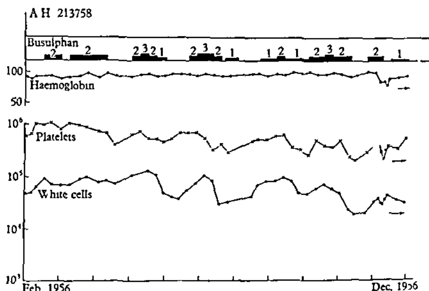
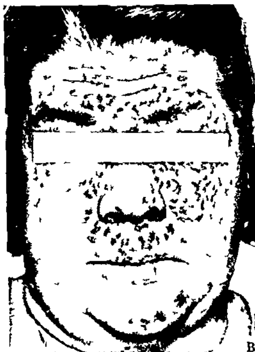


Fig 1 An example of the close relationship of platelets and granulocytes in chronic myelocytic leukaemia (Leucocytes and platelets on a three-cycle logarithmic scale and haemoglobin (% Haldane) on a single logarithmic scale are plotted against time in months on a linear scale Daily dosage of busulphan is recorded as numbers of 2 mg tablets)

Both clinically and histologically follicular lymphoma provides many examples of the type of transition from one type of cell proliferation to another which is illustrated in Plate 2 A–C In this patient the initial biopsy (Plate 2 A) showed a characteristic follicular proliferation of lymphocytes, and at this stage the patient was well apart from anatomical lymph node enlargement After a period of years there was a gradual change clinically the patient became ill and anaemic and developed serous effusions Histologically there was also a complete change this is shown in Plate 2 B and C In Plate 2 B a pattern of lymphosarcoma was established while in other lymph nodes (Plate 2 C) transition to a well defined reticulum cell



A Lymphosarcoma in gut In this patient the process was app rently xclusively intra abdom n l (H & E ×28 6)



B Focal proliferation of blast cell in dermis in a pat ent with acute leukaemia

(Fac p 128)

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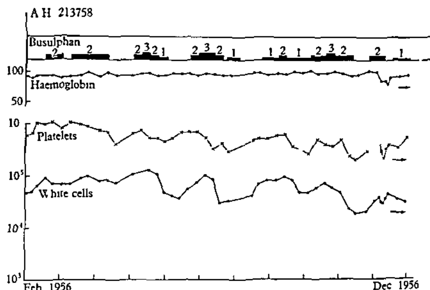
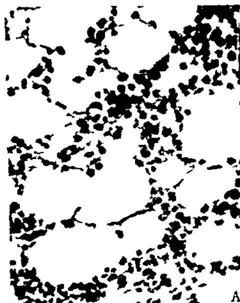


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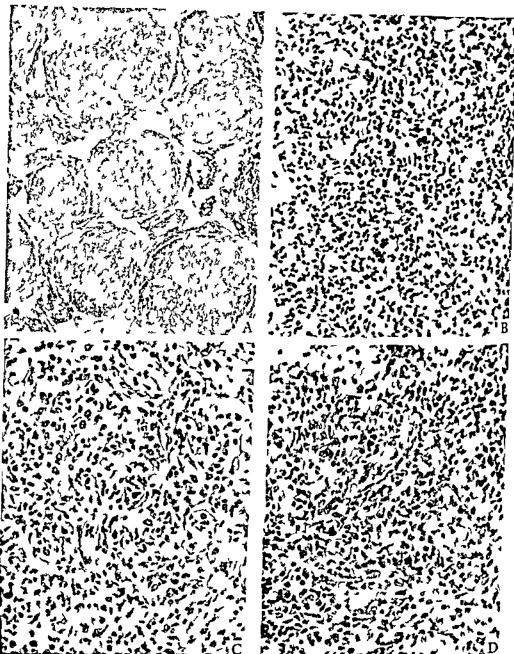
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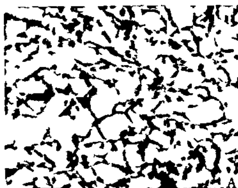
A Initial marrow trephin sho ing hypocellular marrow u th isl nds of blast cells and red cell p ecursors F om the patient whose course i illu trated in F g 3 (H & E ×357)

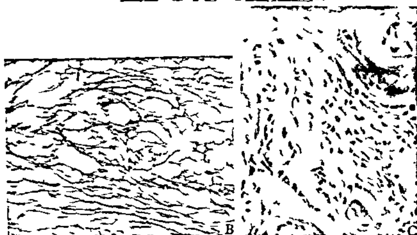


B Fo t mortem hypoc llul ma row from the patient who e course s illu trat d n F g 3 (H & I ×100)



A Follicular lymphoma Initial lymph node biopsy (H & E ×28·6) B Post mortem lymph node from the same patient as Fig A showing transition to lymphosarcoma (H & E ×200) C Post mortem lymph node from the same patient as Figs A and B showing transition to reticulum cell sarcoma (H & E ×200) D Cat scratch fever Lymph node biopsy showing pleomorphic proliferation of lymphocytes and reticulum cells (H & E ×200)





A R b b opsy sh wing pure l ne ret culum c ll prol feration (ee Fig B) (H & E ×357) B R b biopsy (F g A) to show ret culin fibrils (Ret culin ×357) C Rib b op howing almost pure l ne eticulum cell prol ferat on with e rl collagen forma t on (H & E ×200)

PLATE 4



Subcutaneous haemorrhage in a patient with thrombocythaemia

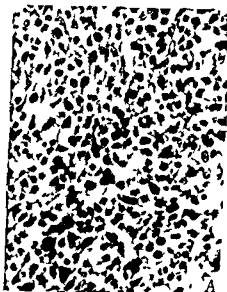
sarcoma was observed Similarly in myelofibrosis a condition which I shall consider in more detail later a wide variety of histological patterns may be observed *in the same patient It would be possible to cite many* other examples of abnormal anatomical presentation and of histological combinations and transitions all of which support the concept of a group of interrelated proliferative processes involving a system of cells of mesen chymal origin

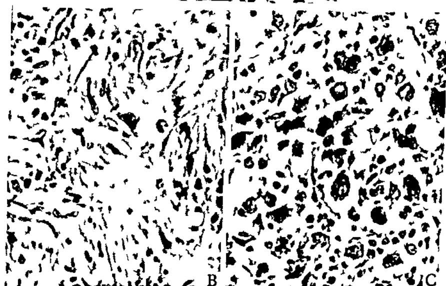
This concept of a group of conditions—the myeloproliferative syndromes —is of practical value and has done much to further understanding and management of the individual members of this group and I personally feel that the term has much to recommend it It is necessary to recognize however that it has limitations and dangers

It is particularly dangerous if in using this term as a practical classifica tion we allow ourselves to believe that it has any aetiological significance This may be made clearer if we first consider a group of conditions which clinically and histologically may present as members of this group but which are aetiologically quite distinct

Cat scratch fever for example may present with enlarged lymph nodes and malaise biopsy (Plate 2 D) may very reasonably suggest a diagnosis of *Hodgkin s disease which may only be disproved by a positive skin test* A similar condition which clinically and histologically may be indistinguish able from Hodgkin s disease is found in patients treated with mesantoin and similar anti convulsant drugs The histological findings in patients with torulosis and glandular fever may again closely resemble those found in patients with Hodgkin s disease The difficulty which competent histologists may experience in distinguishing these conditions emphasizes *the point that many disorders which we now confidently classify as* myeloproliferative may though histogenetically related be aetiologically quite distinct

While it has been traditional to regard for example the leukaemias as a composite group having a common but unknown aetiological mechanism it seems perfectly reasonable to suggest that chronic myelocytic leukaemia is a conditioned proliferation of granulocytes that only the acute myelo blastic *form is a true autonomous proliferation and that the relationship between these two processes is similar to the conditioned and* autonomous pituitary tumours produced experimentally by Furth (1953) This hypo thesis gains some support from the recent demonstration that chromosome abnormalities have so far been found only in the acute blast cell leukaemias (Jacobs 1959) and from the observation that the proliferation observed in granulocytic leukaemia may be modified by some factor present in normal plasma (Schulz & Florian 1954–5 Schoyer 1959)





A Marrow from a patient with myelofibrosis Other sections from the *same* patient are shown in Figs B and C This section is from an area with a predominant proliferation of granulocyte precursors (H & E ×357) B Marrow from a patient with myelofibrosis Other sections from the *same* patient are shown in Figs A and C This section is from an area with predominant proliferation of reticulum cells with considerable collagen formation (H & E ×357) C Marrow from a patient with myelofibrosis Other sections from the *same* patient are shown in Figs A and B This section is from an area with a predominant proliferation of multinucleate reticulum (megakaryocyte like) cell (H & E ×357)

Table 2

Lymphocyte	Granulocyte
Lymphocytic leukaemia	Myelocytic leukaemia
Lymphoblastic leukaemia	Myeloblastic leukaemia
Follicular lymphoma	
Lymphosarcoma	Monocyte
	Monocytic leukaemia
Red cell	Plasma cell
Polycythaemia vera	Myeloma
Erythroleukaemia	
Platelet	Reticulum cell
Thrombocythaemia	Reticulum cell sarcoma
Mixed	
Hodgkin s	
Myelofibrosis	
Pre leukaemia	

The conditions which I should now like to consider in greater detail are (1) preleukaemia (2) a syndrome intermediate between aplasia and leukaemia (3) thrombocythaemia and (4) the group of conditions in which proliferation of reticulum cells in the marrow is the predominant feature

PRE LEUKAEMIA

It is perhaps improper to regard this condition as a myeloproliferative syndrome but it deserves some consideration in the present context It may be defined as an abnormal haematological state in which thorough investigation reveals no evidence or suggestion of leukaemia but which after a variable period of time undergoes transition to an unequivocal leukaemic state It is important to exclude from this group those conditions which although presenting for example as refractory or aplastic anaemia show early evidence of leukaemia such as a small percentage of blast cells in the marrow This condition which has attracted increasing interest in the last five to ten years, is of considerable practical and theoretical importance

Table 3 summarizes the relevant details in a number of cases observed by ourselves (Richardson & Wetherley Mein 1959) or described in the literature (Hamilton Paterson 1949 Block Jacobson & Bethard 1953 Bernard & Boiron 1954 Meacham & Weisberger 1954 Williams 1955 Blackburn 1957 Rowen 1957) All these cases satisfy the criteria which I have mentioned You will see that in the so called preleukaemic phase the clinical and haematological diagnosis based on physical examination peripheral blood counts and marrows was varied, although the majority of patients were justifiably diagnosed as having aplastic anaemia with hypo cellular marrow You will also see that with few exceptions all these

Certainly the sharp contrast between the benign and easily controlled chronic myeloid leukaemia and the almost unmanageable myeloblastic state suggests that some fundamental change has occurred It is tempting to suppose that were it not for this change, possibly a mutation patients with chronic myeloid leukaemia could be maintained indefinitely either by suppressive chemotherapy or some sort of replacement therapy

With other leukaemias, particularly those affecting the lymphoid series, it has been suggested that they may, in the chronic stage at any rate represent a disturbance of immune mechanisms (Damashek & Schwartz 1959) and recent speculation based chiefly on the clinical changes found in animals with runt disease has raised the possibility of a similar aetiology for Hodgkin s disease (Kaplan & Smithers 1959)

Although there is, as yet, no evidence which establishes these specula tions on the nature of the chronic leukaemias or Hodgkin's disease the position of polycythaemia vera has recently become more sharply defined This condition is frequently associated with thrombocythaemia myelo fibrosis, and a granulocytic proliferation which, except in terms of leuco cytic alkaline phosphatase, closely resembles chronic myeloid leukaemia and may terminate by transition to a blast cell leukaemia

For these reasons it has been very properly accepted as a member of the myeloproliferative group in which differentiation is predominantly in the direction of the red cell Recent studies on erythropoietin, an erythro poieticstimulatingfactor (Bethell, Linman & Korst, 1957, Payne, 1959) have established that this substance is not only present in normal plasma where it presumably exists as part of the physiological homeostatic mechanism controlling erythropoiesis but that it is also present in considerable excess *in the plasma of patients with polycythaemia vera* Although this finding may be interpreted in several ways the most probable explanation is that the excessive proliferation of red cells is not autonomous but is conditioned by and dependent on an excessive production of this normal circulating substance The significance of these findings in an established member of the myeloproliferative group of disorders cannot be determined without further clinical and experimental study Although it would be unwise to extrapolate the findings in polycythaemia to histogenetically related condi tions the possibility that they are all proliferations conditioned by distinct stimuli is at least worth disproving

While it is clear therefore that this concept has limitations particularly in terms of aetiology it has on other grounds much to recommend it as a practical working hypothesis The conditions which may reasonably be classified as myeloproliferative syndromes are according to *line of dif ferentiation*, shown in Table 2

The theoretical significance of this relatively uncommon condition may be briefly stated by asking the question Are these patients leukaemic from the start or is any patient with prolonged haematological abnormality more likely to develop the mutation which is presumably the change which determines transition to acute leukaemia? It is obviously impossible to answer this question but I think it not unlikely that a variety of abnormal stimuli which include ionizing irradiation infection or the existence of some unrelated haematological abnormality may be among the triggering factors which determine the development of acute leukaemia

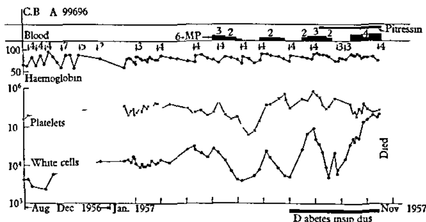


Fig 2 Haematological findings in a patient presenting with refractory anaemia and terminating w th a blast-cell leukaemia (Leucocytes and platelets on a three-cycle logarithmic scale and haemoglobin (Hald ne) on a single logarithmic scale are plotted against time in months on a linear scale D ily do age of 6 MP is recorded as numbers of 50 mg tabl ts)

A SYNDROME INTERMEDIATE BETWEEN APLASTIC ANAEMIA AND LEUKAEMIA

A condition which appears to be in many ways intermediate between aplastic anaemia and leukaemia is that illustrated by the patient whose findings are shown in Plate 3 A B and Fig 3 He presented with a pancytopenia and no abnormal physical signs and no immature cells in his peripheral blood His marrow aspirate was rather less cellular than normal and contained almost equal numbers of leucoblasts and red cell precursors The marrow trephine (Plate 3 A) showed a hypocellular rather fatty marrow with islands of blast cells and nucleated red cells As shown in Fig 3 this patient was treated and indeed behaved as an aplastic anaemia At no time was there any splenomegaly or peripheral evidence of leukaemia and he died as other patients we have observed with this condition of

patients developed an acute blast cell leukaemia The time interval between the initial diagnosis and the development of a leukaemia varied considerably and in a number of patients there was an intervening period of complete return to clinical and haematological normality

Table 3 *Pre leukaemia*

No of cases	Initial diagnosis	Interval (months)	Remission (months)	*Final diagnosis*	Age *(years)*
11	Aplastic anaemia Pancytopenia	Mean 13 Range 1½–84	4 patients 1–54	Acute leukaemia	Mean 50 Range 2½–75
6	*Megaloblastic anaemia*	Mean 60 Range 1–296	—	Acute leukaemia (3) Myelocytic leukaemia (3)	Mean 52 Range 25–68
2	Acquired *haemolytic anaemia*	2 9	*1 patient* 1	*Acute leukaemia*	4 27
2	Red cell aplasia	15 48	—	Acute leukaemia (1) *Myelocytic leukaemia* (1)	47 55
2	Drug pancytopenia	5 3	1 patient 4	Acute leukaemia	36 50
2	Agranulocytosis	12 7	1 patient 5	Acute leukaemia	1½ 61
1	*Splenic pancytopenia*	18	—	Monocytic *leukaemia*	56
1	Non thrombocytopenic purpura	27	—	Acute leukaemia	50

The findings in one of our patients who fell into this category are shown in Fig 2 In 1954 two years before she presented to us in 1956 she had been diagnosed as a refractory anaemia with hypocellular marrow there was no reason to alter the diagnosis She was maintained solely by trans fusion and remained well In February 1957, however, occasional immature leucocytes appeared in the peripheral blood and during the next two months there was increasing evidence based on peripheral blood and bone marrow examination that she was developing acute leukaemia This *was only indifferently controlled by 6 Mercaptopurine and she eventually* died approximately three years after the onset of her refractory anaemia The post mortem confirmed the diagnosis of acute leukaemia and established incidentally that her diabetes insipidus was associated with leukaemic involvement of the pituitary

In the majority of cases it is obviously impossible to predict or even to suggest that one is dealing with a patient in a preleukaemic state It is however reasonable to bear in mind that any patient with a prolonged refractory anaemia with or without acellular marrow may undergo transi tion to leukaemia In particular in a patient with a megaloblastic anaemia relapse on adequate therapy following an initial satisfactory response to B_{12} or folic acid *should suggest the possibility that leukaemic transition is* occurring

and in those who like this lady we have treated with busulphan, a satisfactory fall in the platelet count has been associated with cessation of the haemorrhagic tendency (Edgcumbe & Wetherley Mein 1950).

The mechanism of bleeding in these patients is not clear. Characteristically their first complaint may be of small painful nodules in the subcutaneous tissue, initially colourless and later developing an areola of extravasated blood. There is evidence that the functional efficiency of both

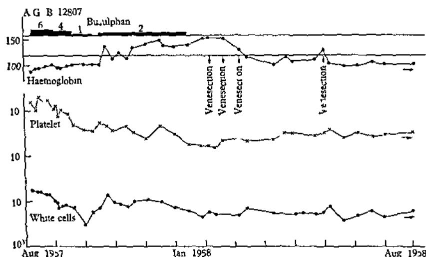


Fig 4 Haematological findings in the patient with thrombocythaemia whose presenting syndromes are illustrated in Plate 4 (Leucocytes and platelets on a three cycle logarithmic scale and haemoglobin (% Haldane) on a single logarithmic scale are plotted against time in months on a linear scale.) Daily dosage of busulphan is recorded in mg.

normal and thrombocythaemic platelets is impaired when they are in the high concentrations in which they are found in this condition and that a return to normal function occurs when the platelet count is reduced either *in vitro* or *in vivo*, to normal levels (Ingram & Glass 1959). From a practical point of view the possibility of thrombocythaemia should be considered in any patient showing an abnormal bleeding tendency and particular care should be taken to avoid operation in such patients for they almost invariably bleed excessively. Treatment with busulphan seems in our experience to be effective and preferable to the use of radioactive phosphorus.

pneumonia The post mortem showed no evidence of leukaemia but the marrow (Plate 3 B) was still hypoplastic and blastic

This syndrome, which might be called either atypical leukaemia or atypical hypoplasia is worth recognizing, for these patients appear to do reasonably well if treated as aplastic rather than leukaemic and its existence may be suspected in patients with all the clinical and peripheral evidence *of aplastic anaemia who are found to have hypocellular* marrow with a high proportion of blast cells

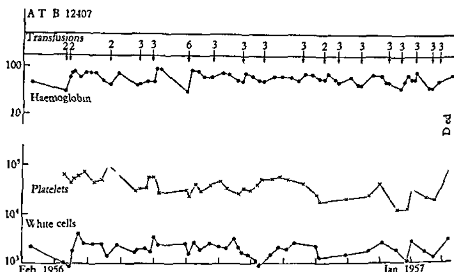


Fig 3 Haematological findings in a patient with a syndrome intermediate between aplastic anaemia and leukaemia (see Figs A and B) (Leucocytes and platelets on a three cycle logarithmic scale and haemoglobin (° Haldane) on a single logarithmic scale are plotted against time in months on a linear scale)

THROMBOCYTHAEMIA

The next condition I should like to consider is thrombocythaemia This is characterized by a proliferation of megakaryocytes associated with a considerable rise in the circulating platelet count Clinically the most common presentation is bleeding either gastro intestinally or as a subcutaneous extravasation Such a situation is illustrated in Plate 4 where as you will see there was massive extravasation of blood into abdomen thigh and arm The haematological findings and the course of this patient are fairly characteristic of this condition and are shown in Fig 4 At the outset there was a platelet count of 1 600 000 with a white count only raised slightly above normal Approximately half these patients have a negative leucocyte alkaline phosphatase and in approximately the same number the spleen is palpable the platelets themselves are morphologically abnormal

course with progressively unmanageable anaemia and commonly terminate in cardiac failure or occasionally by transition to acute blast cell leukaemia Their response to chemotherapy radiotherapy and steroids is usually very disappointing and progressive shortening of red cell survival sooner or later makes transfusion ineffective At this stage splenectomy which in some patients may be followed by a dramatic rise in red cell production and a lengthening of red cell life span may transform the situation and offer the patient a further span of worth while existence

SUMMARY

An attempt has been made to re examine the concept of the group of related disorders which may be regarded as myeloproliferative syndromes It still seems reasonable on grounds of common histogenesis to accept this concept as the basis of a practical working classification There are how ever growing indications that many members of this group which includes the acute and chronic leukaemias the lymphomas and the myelo fibrotic syndromes may be aetiologically quite distinct It seems probable and indeed it is to be hoped that with further understanding of pathogenesis this group will disintegrate and be replaced by a number of distinct processes of specific aetiology

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PROLIFERATION OF RETICULUM CELLS IN MARROW

Finally, I should like to consider the syndromes in which proliferation of reticulum cells in the marrow is the predominant feature These conditions variously called myelofibrosis, myeloid metaplasia megakaryocytic myelosis and reticulum cell sarcoma are most profitably grouped as myelopro liferative syndromes because the clinical, haematological and histological presentations are so varied and often so closely overlap that precise classification in the individual patient is often impossible and may indeed obscure the fact that the subsequent clinical course and response to treat ment are remarkably similar

Clinically these patients most commonly present with symptoms of anaemia due, predominantly, to erythropoietic failure but often with an associated haemolytic process, rarely of auto immune type Occasionally polycythaemia rather than anaemia may be present The spleen may be enormous or impalpable and in the peripheral blood the pictures of pancyto penia, thrombocythaemia or myelocytic leukaemia may be observed

The histological features are also extremely varied not only between patients but also in individual patients This variability which resembles that seen in the later stages of follicular lymphoma again emphasizes the versatility of mesenchymal tissue and is illustrated in Plates 5 and 6 The patient whose marrow biopsies are shown in Plate 5 A and B presented with erythropoietic failure a moderately increased leucocyte count with atypical immature mononuclear cells and a reduced platelet count The marrow aspirate was very hypocellular and contained scattered reticulum type cells The rib biopsy (Plate 5 A) showed a diffuse marrow replacement by an almost pure line reticulum cell proliferation with as shown in Plate 5 B dense reticulin formation Plate 5 C shows another rib biopsy from a patient presenting with pancytopenia no splenomegaly and an acellular marrow aspirate Here there is again a pure line reticulum cell proliferation with dense reticulin and early collagen formation Both these conditions could be classified as reticulum cell sarcoma of the marrow More complex patterns produced by the apparently simultaneous proliferation of reti culum cells and granulocytes are seen in Plate 6 A–C which all come from a patient who presented with gross splenomegaly a leuco erythroblastic blood picture and again an acellular marrow aspirate Here almost every pattern of abnormality seen in the condition which is usually called myelo fibrosis or myeloid metaplasia can be observed For reasons which I have previously discussed it is unprofitable to consider the relationship of these conditions in terms of aetiology In terms of clinical behaviour and response to treatment they resemble each other in so far as they generally run a slow

EVIDENCE FOR INCREASE IN INCIDENCE

No data are available for morbidity over any great length of time but, in view of the high fatality of the disease it is reasonable to use mortality statistics as indicators of incidence

In England and Wales the mortality attributed to leukaemia has increased from 19 per million men and 15 per million women in 1931 to

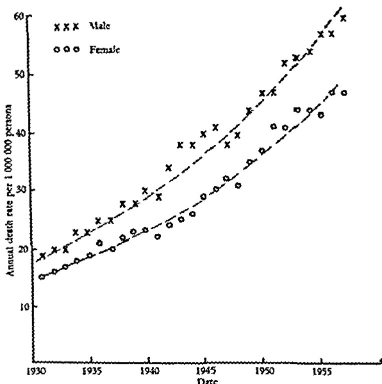


Fig 1 Leukaemia mortality in England and Wales from 1931 to 1957 crude death rates per million persons shown separately for men and for women.

60 per million men and 47 per million women in 1957* (Fig 1) Throughout this period the rate of increase has been fairly steady and has approximated to 4 4 % per annum that is the mortality has doubled every sixteen years The rate of increase has been the same for men and for women and in this country has been greater than that for any major cause of death other than cancer of the lung and coronary thrombosis

* The Registrar General has corrected the classification employed for aleukaemic leukaemia before 1938 and the figures quoted include deaths attributed to this cause

THE INCIDENCE OF LEUKAEMIA AND THE SIGNIFICANCE OF ENVIRONMENTAL FACTORS

RICHARD DOLL

Knowledge of the incidence of leukaemia is, at present so incomplete that any conclusions based on differences in the incidence recorded under different conditions are of necessity, speculative Two limitations in particular, must be recognized First the diagnosis of the disease is relatively sophisticated and it cannot be assumed that the same diagnostic standards have been employed at different periods, in different countries or even in different areas and among different social classes within the same country Until 1938 for example aleukaemic leukaemia was not classified with leukaemia in the International List of Causes of Death but was grouped with Hodgkin s disease and presumably this must have reflected earlier haematological opinion More recently, we have seen a great expansion of the pathological services and it is difficult not to believe that the influx of clinical pathologists into the more peripheral hospitals has not had the effect of increasing the facility with which the disease is diagnosed in the outlying areas Secondly there is the possibility that leukaemia is not one disease, but that the individual cytological and clinical types represent different disease entities with independent causes On present knowledge it seems probable that leukaemia comprises at least three separate diseases—chronic lymphatic leukaemia, chronic myeloid leukaemia and acute leukaemia—and it is possible that acute leukaemia should be further divided In the present paper the three main types of leukaemia will be considered separately whenever possible but suitable data are not always available and it is often necessary to study the incidence of leukaemia as a whole

It is therefore clearly hazardous to lay any great weight on the differences which have been recorded in the incidence of leukaemia Never theless in the absence of more certain evidence it is I believe useful to examine the available statistics—but it is important to remember that with few exceptions the clues provided may be false

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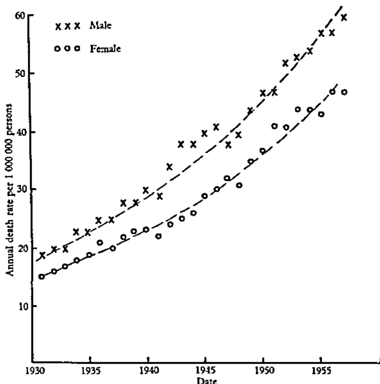


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The rate of increase has, however, not been the same at all ages From 1931 it was, at first, greater at ages three and four years and at ages over sixty years than at other ages since 1950 the increase has been almost confined to the older age groups and there has been no increase at all at ages under fifteen years (Fig 2) When the various types of leukaemia are considered separately it seems probable that the small recent increase in early adult life and middle age has been confined to acute leukaemia except perhaps for some small increase in chronic myeloid leukaemia in men There is however no evidence of any increase in chronic lymphatic leukaemia under the age of sixty years Above sixty years the

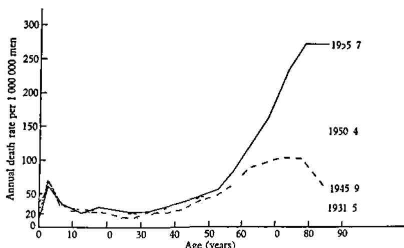


Fig 2 Leukaemia mortality among men in England and Wales 1931–5 to 1955–7 by age

increase has been substantial and has been shared by all the types (Court Brown & Doll 1959) These data could not be obtained directly from the national mortality data as until recently a substantial proportion of the deaths had been attributed to lymphatic leukaemia myeloid leukaemia or even leukaemia alone without further specification They have therefore had to be estimated by allocating the unspecified deaths to the individual types of leukaemia by assuming that at each age the unspecified cases included the various types in the same proportions as occurred when the types were specified The conclusions are therefore of necessity tentative How much of the recent increase is real is impossible to say but if the increase in early adult life and middle age is confined to one specific type it would seem reasonable to believe that this increase might well be real In contrast the very large increase at ages over sixty years is too great to be regarded as a cohort effect—at no time in the past has there been a cor

respondingly rapid increase in mortality at younger ages—and since it is shared by all the types it seems reasonable to believe that much of it is spurious and due to a more complete recognition of the disease in this age group That greater precision in diagnosis has recently been obtained for the older age groups is clearly demonstrated by the rapid fall that has occurred in the last decade in the mortality attributed to senility

AGE DISTRIBUTION

One of the most striking epidemiological characteristics of leukaemia is the shape of the curve relating mortality to age Unlike most other types of cancer this curve shows two clear peaks the first at ages 3–4 years and the second in old age When this type of curve does occur the explanation is invariably found to be that the two peaks are produced by two histological types which although arising in the same organ are quite distinct—one type for example arising from mesoderm and the other from ectoderm This explanation does not appear to hold for leukaemia The chronic types show only the peak in old age but the double peak persists in acute leukaemia (MacMahon & Clark 1956 Court Brown & Doll 1959) It may be therefore that acute leukaemia includes at least two different diseases one of which has a maximum mortality in early childhood while another has a maximum mortality in old age

Close examination of the mortality by single years of age suggests that there may be a third but less important peak in adolescence This was noted by Lee (personal communication) who found it to be present in the British data for the years 1945–9 and 1950–7 and in the U S A data since 1940 Earlier data are not available in sufficient detail for the presence of the peak to be looked for at other periods Lee found that the peak occurred at about age 16–17 years and that it was present only in males

Analysis of the childhood peak has revealed some interesting facts which may provide clues to the aetiology of the type of disease occurring at this age Burnet (1958) examined the data for the U S A and found that the three year old peak of mortality was a relatively new development When he examined the mortality of children born in 1939 he found that the peak mortality was only slightly greater than that for other ages in childhood and that it occurred at age two years In contrast children born in 1945 or subsequently showed a sharp peak of mortality at age three years Between 1939 and 1945 there was a gradual change from one type of curve to the other and Burnet concluded that some leukaemogenic agent was introduced into the U S A in the early 1940 s which affected children *in utero* or at the latest during their first year of extra uterine life British data also show

a similar change from a small two-year old peak to a sharp three year old peak, but in this case the change appears to have occurred in children born between the years 1934 and 1936 (Fig 3) It is difficult to see how these changes in the age distribution of mortality could be artefacts and it seems reasonable to presume that the relevant leukaemogenic agent was introduced into Britain some 5–7 years before it was introduced into the U S A

Two other points of possible importance are (1) that in the U S A the childhood peak is limited to the white population and does not occur among

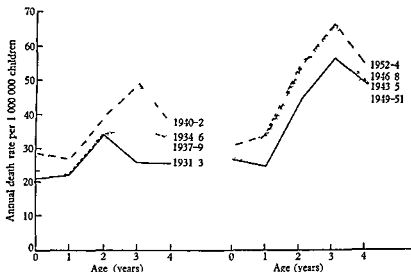


Fig 3 Leukaemia mortality at ages 0–4 years for cohorts of children born in England and Wales in three year periods from 1931–3 to 1952–4

Negroes (Walter & Gilliam 1956) and (2) that in Britain the peak is sharper for first born children than for the later members of a family (Stewart Webb & Hewitt 1958)

All types of leukaemia contribute to the second peak in old age but the mortality from chronic lymphatic leukaemia increases with age more rapidly than the mortality from the other types and, as age increases it comes to play an increasingly predominant part In the case of acute leukaemia and chronic myeloid leukaemia the rate of increase with age is substantially less than that for most other cancers but it is notable that the estimated mortality from chronic lymphatic leukaemia increases approximately in proportion to the sixth power of the age and in this respect is comparable with the principal types of epithelial cancer other than those that are hormone dependent

SEX RATIO

The sex ratio (male female mortality rates) is higher for chronic lymphatic leukaemia than for either of the other types For chronic lymphatic leukaemia it is approximately 2 1 at all ages For chronic myeloid leukaemia it is about 1 2 1 For acute leukaemia it is somewhat higher (about 1 4 1) and is at a maximum at those ages at which the mortality is also at a maximum

VARIATION BETWEEN COUNTRIES

The death rates from leukaemia recorded in twenty six countries and standardized for age are shown in Table 1 There is a ninefold difference between the highest and lowest rates but it is likely that much of this is artificial and due to incomplete recognition of cases in countries with a very low recorded mortality It is however unlikely that all the differences are spurious There is no reason to suppose that leukaemia is recognized more readily in Denmark and among the white population of the U S.A than in Germany Britain and Belgium and the high mortality in the former countries is likely to reflect an unusually high incidence of the disease

Table 1 *Leukaemia mortality in different countries standardized death rate per 1 000 000 men 1952–6*

Country	Death rate	Country	Death rate
U S A (white population)	82	U S A. (non white population)	53
Denmark	80	England and Wales	53
Israel	72	Finland	52
Norway	71	Scotland	52
Sweden	70	*Belgium*‡	52
New Zealand*	69	Italy	49
Canada	65	Eire	41
Switzerland	63	Portugal§	32
Austria†	62	Chile‡	30
Holland	62	Japan	25
Australia	58	Venezuela	21
France	57	Colombia†	15
West Germany	56	Ceylon	9

* 1952–5 † 1953–6 ‡ 1954–6 § 1955–6

The low mortality in Japan is due almost entirely to a deficiency of deaths at the older ages Under forty years of age the Japanese mortality is practically the same as that recorded in Britain but after ages 55–59 years the Japanese mortality begins to fall off and by ages 70–79 years the British mortality is approximately eight times the Japanese (Fig 4) This may be partly due to an incomplete recognition of cases at the older ages in Japan (old people are not covered by an insurance system and they are less inclined

to seek hospital attention) but it is partly due to the great rarity of chronic lymphatic leukaemia The infrequency of this disease in the Far East has been confirmed by the studies of the American Atomic Bomb Casualty Commission on Hiroshima residents who were not exposed to the effects of the bomb and by clinical studies on groups of patients in several other eastern countries

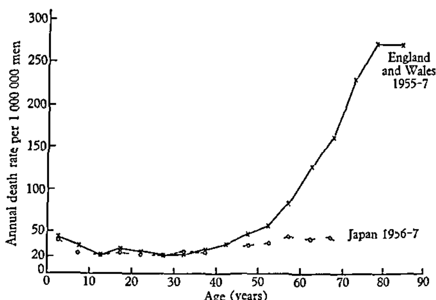


Fig 4 Comparison between mortality attributed to leukaemia among men in England and Wales (1955–7) and in Japan (1956–7) in five year age groups Japanese data reported by Segi (1959)

VARIATION WITHIN BRITAIN

Hewitt (1955) examined the mortality data for leukaemia in different parts of Britain during the four years 1950–3 and found no consistent differences between the mortality in urban and rural areas There was however, a fairly regular gradient in mortality from a low level in the north of England and in Wales (standardized mortality ratios of 83 and 89) to a high level in the south and south-eastern regions (S M R s of 121 and 114) Within London the highest mortality was recorded in the four contiguous and wealthy boroughs of Chelsea Westminster St Marylebone and Hampstead (S M R 185) The regional variation was similar for both sexes but was much more marked for the older age groups than for the younger In London for example the mortality at ages over seventy five years was double the national average whereas at ages under forty five years it was practically normal

In a more recent study Phillips (1959) has shown that the southern excess persisted in the period 1954–7 He was however particularly struck by the fact that the rate of increase in the crude mortality between 1950–3 and 1954–7 had been greatest in five predominantly rural counties situated in the north of England or Wales (Cumberland Westmorland Caernarvon shire Montgomeryshire and Monmouthshire) In all these counties the increase had been over 50% compared with a national average of 13% Phillips pointed out that four of these counties were predominantly mountainous with a high rainfall and he suggested that the greater increase in these areas might be attributed to the greater deposition of radioactive material from the atmosphere—particularly of radioactive strontium This view is however not tenable because the quantities involved would not on any reasonable hypothesis account for the extent of the increase (Loutit 1959)

It should moreover be noted that four of the five counties referred to had very low death rates in the earlier period and that the increase in mortality in these counties brought their death rates close to the national average Low mortality rates due to random factors would not be expected to persist in subsequent periods and chance factors alone could be expected to account for the biggest increases being observed in areas with initially the lowest mortalities It is possible also that the development of the patho logical services since 1948 has contributed to the greater recognition of leukaemia in areas which were previously poorly served—and if this is so it is just the mountainous rural areas which would be most likely to be affected

VARIATION WITH SOCIAL CLASS

In an analysis of occupational mortality the Registrar General (1938) found that in 1930–2 the mortality from leukaemia and Hodgkin s disease com bined was substantially higher among the wealthier sections of the popula tion of England and Wales (social classes I and II) than among the poorer (social classes IV and V) This was true for both men and married women Twenty years later the differential for both diseases was less marked (Table 2) Analysis by age showed that an excess mortality from leukaemia among the wealthier classes still occurred among men at the older ages but there did not appear to be any substantial difference for the different age groups among women (Table 3) MacMahon (1957) also found an associa tion between high incomes and leukaemia mortality in an analysis of mortality data for the various States of the U S A the association was however less marked than that found between leukaemia and the density of physicians It is not clear whether the type of difference noted can best

be accounted for by different standards of diagnosis or by different exposure to leukaemogenic stimuli—including, perhaps, some associated with medical attention

Table 2 *Leukaemia mortality by social class, standardized mortality ratios England and Wales, 1930–2* and 1949–53 (Registrar General, 1958)*

Social class	Men		Married women	
	1930–2	1949–53	1930–2	1949–53
I	153	123	167	145
II	125	98	118	92
III	96	104	107	102
IV	94	93	76	104
V	85	89	76	87

* Deaths from Hodgkin's disease are included with leukaemia in the 1930–2 classification but the comparison with 1949–53 is justified in that the two conditions are correlated similarly with social class in the later period

Table 3 *Leukaemia mortality by social class and age standardized mortality ratios England and Wales 1949–53*

Social class	Men (years)			Married women (years)		
	20–34	35–54	55–64	20–34	35–54	56–64
I	67	127	144	126	158	135
II	89	89	112	89	96	88
III	103	101	108	99	96	113
IV	96	94	91	107	108	99
V	107	103	72	104	88	79

Table 4 *Leukaemia mortality by occupation men aged 20–64 years England and Wales 1949–53*

Occupation	No of deaths	Standardized mortality ratio
Makers of coal gas and coke	11	275
Railway officials	11	220
Electrical fitters	21	210
Armed forces officers	18	200
Royal Navy other ranks	14	200
Locomotive drivers and motormen	22	169
Printers and bookbinders	33	150
Coal miners (underground not at face)	30	67
Building workers labourers	23	39

VARIATION WITH OCCUPATION

Even in a five year period the number of leukaemia deaths among men actively employed in a given occupation is small and the Registrar General's occupational mortality data do not provide clear cut evidence for the presence or absence of any specific industrial risks Some of the unusually high and low mortalities are summarized in Table 4 In view of the experi

mental production of leukaemia by the injection of some polycyclic hydro carbons it is perhaps interesting to inquire whether a similar effect may be produced among gas workers by exposure to fumes from coal tar

Data from the U S A provide evidence of an occupational risk among radiologists The data are summarized in Table 5 March (1947 and 1950) analysed the causes of death of doctors recorded in the obituary columns of the *Journal of the American Medical Association* and found that the proportion attributed to leukaemia was some nine times higher among radiologists (14/299) than among other doctors (334/65 922) This evidence was strengthened by Dublin & Spiegelman s (1948) finding that the death rate from leukaemia among radiologists was higher than the rates estimated for other specialists and for other doctors on the files of the American Medical Association

Table 5 *Leukaemia mortality among radiologists*

Country (reference)	No of d aths	Estimate of no expected	Basis for estimate
U S A (Dublin & Sp egelman 1948)	5	0 8	Mortality rate of all doctors
U S A (March 1950)	14	1 5	Proportional mortal ty of other doctors
B itain (Court Brown & Doll 1958)	3	1 4	Mortality rate of men in social class I

Data for British radiologists (obtained by following up all the medical members of the British Institute of Radiology the Faculty of Radiologists and their antecedent societies from the foundation of the Roentgen Society in 1897 to 1 January 1957) failed to show any substantial risk of the disease (Court Brown & Doll 1958) The discrepancy may however be less real than apparent as there is reason to believe that British radiologists made use of protective measures on a large scale earlier than their American colleagues

Browning (1958) is of the opinion that chronic exposure to benzene may provoke leukaemia Several cases have been observed in workers exposed to benzene and some appear to have developed out of a preceding aplastic anaemia No data are however available to enable the incidence in benzene workers to be compared with that which occurs normally and benzene cannot be regarded as a proven cause of the disease

IONIZING RADIATIONS

There is no doubt that ionizing radiations are one cause of leukaemia This has been demonstrated in experimental animals is supported by the mortality data for American radiologists and is confirmed by the experience

of survivors of the atom bomb explosions at Hiroshima and Nagasaki and of patients who have been irradiated for ankylosing spondylitis Among the Japanese who were exposed at less than 1000 metres from the hypocentre of the explosion risk of developing the disease has been increased approximately hundred fold (Medical Research Council 1956) among some 13 000 irradiated patients it has been increased ten fold (Court Brown & Doll 1957) It is certain also that the latent period following exposure before the appearance of the disease is commonly between three and eight years but a few cases appear earlier and the risk does not disappear entirely by the end of the twelfth year Both acute leukaemia and chronic myeloid leukaemia are produced by irradiation but acute leukaemia is the most characteristic type At Hiroshima, however the two types were produced with almost equal frequency The reason for this discrepancy is not known it may perhaps depend on some peculiarity in the conditions of irradiation at Hiroshima There is, so far, no evidence to suggest that chronic lymphatic leukaemia can be induced by irradiation No excess has been observed within twelve years of exposure, so that if it is induced in this way, the latent period must be much longer than for the other types of the disease

The demonstration of an excess incidence of leukaemia following irradiation has, so far, been confined to observations on high doses (of the order of 100 rad or more) given at rates of at least 10 rad/min This is not surprising as the quantitative relationship between the dose of radiation and the subsequent incidence of the disease is such that immense numbers would have to be examined before any effect from small doses could be observed That the amounts of radiation employed in diagnostic radiography may also induce the disease is however suggested by several studies in which the histories of patients with leukaemia have been compared with those of other non affected subjects Faber in Denmark compared the frequency with which X ray examinations were noted in the previous records of patients who developed leukaemia with that noted in a control group and found evidence of more exposure to radiotherapy and to diagnostic X ray examinations of the trunk in patients with acute leukaemia or chronic myeloid leukaemia than in patients with chronic lymphatic leukaemia or in the control group (Faber 1957 Faber Andreasen & Uhrbrand 1958) Stewart Webb & Hewitt (1958) found a similar type of difference between the past experience of children who died of leukaemia or other malignant disease and a control group of living children born in the same area at about the same time About twice as many of the children who died of malignant disease as of the control children had been exposed to diagnostic irradiation whilst *in utero* The proportion exposed was however, small (about 14% in all) and it is not possible to explain the

three year-old peak in mortality or the main part of the rise in childhood mortality by the increased use of X rays in obstetrics The histories were in each case obtained from the mothers and there is a possibility that some (and perhaps much) of the excess exposure may be spurious and result from a difference in the efficiency of the memory of those mothers who had lost children and those who had not

Results similar to those of Stewart and her colleagues have been obtained in two smaller American studies (Ford Paterson & Treuting 1959 Polhemus and Koch 1959) Contrary results have however been obtained

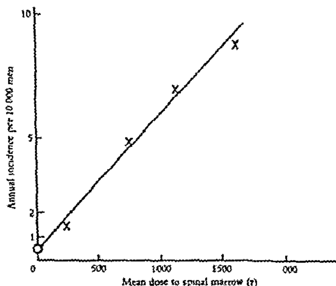


Fig 5 Relationship between incidence of leukaemia standardized for age and mean dose of radiation to the spinal marrow among patients treated for ankylosing spondylitis by irradiation limited to the spine (after Court Brown & Doll 1957)

by MacMahon (1958) this may reasonably be attributed to chance since the number of cases studied was small but it should be noted that his results cannot have been biased by knowledge of the fate of the child since the source from which information about exposure to X rays was obtained was the previous records of the hospital in which the baby was born

Other evidence relating to the effect of diagnostic radiography may be obtained from the shape of the dose response relationship at higher doses Uncertainty about the extent to which the survivors of the Hiroshima and Nagasaki explosions were shielded makes it difficult to derive any exact mathematical relationship between dose and incidence but the present findings are believed to be consistent with a linear relationship Court

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became more prevalent in Britain in 1934–6 and in the U S A in 1943–4 coloured children in the U S A are not exposed to it

(5) Ionizing radiations are one cause of acute leukaemia and of chronic myeloid leukaemia when given in therapeutic doses and probably also when given in diagnostic doses background radiation may be a cause but this is open to question

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Brown & Doll (1957) reached the same conclusion from studying patients who had been irradiated for ankylosing spondylitis The mean spinal marrow dose received by each patient was estimated from measurements made on a phantom and from the individual radiotherapy records It is reasonable to believe that this provides a fair measure of the total leukaemogenic stimulus for patients in whom the radiotherapy was limited to the spine and the results in these patients are likely to provide the best estimate of the dose response relationship The results obtained are illustrated in Fig 5 quantitatively they suggest that 1 rad averaged over the whole marrow might produce about one case of leukaemia per 1,000 000 persons per year for at least six or seven years The data obtained from Hiroshima and from irradiated patients are therefore consistent with the hypothesis that diagnostic radiography is responsible for some cases of leukaemia but only for a small proportion of the total Whether cosmic rays and other naturally occurring background radiations are also responsible for some cases is more doubtful If the apparent linear relationship is interpreted to mean that leukaemia can be induced by a mechanism analogous to a genetic mutation, it would follow that background radiation would also be likely to produce the disease It should, however be noted that the intensity of background irradiation is several orders of magnitude less than that of diagnostic and therapeutic irradiation (10^{-6} rad/min against 10 to 50 rad/min) and with other mechanisms this difference might be of major importance

CONCLUSIONS

Study of the incidence of leukaemia and of its relation to environmental factors provides a number of potentially useful clues to the aetiology of the disease, but few firm facts It may reasonably be concluded that

(1) The incidence of leukaemia varies in different parts of the world it has increased in Britain and other countries during the last thirty years but less than would appear from mortality records

(2) Leukaemia comprises several separate disease entities

(3) There has been little if any real increase in the incidence of chronic lymphatic leukaemia The sex ratio of chronic lymphatic leukaemia is higher than for the other principal types of the disease the mortality increases more rapidly with age and the rate of increase is comparable to that found with the majority of epithelial cancers the disease is relatively rare in the Far East

(4) Acute leukaemia may itself comprise several distinct entities One type occurring in childhood may be attributed to a factor to which the child is exposed *in utero* or at the latest in the first year of life this factor

become manifest The pharmacological properties of the anti leukaemic drugs do not require the use of dose schedules involving high loading doses or alternatively building up to a maximum dose from a small initial dose

THE BASIS OF CHEMOTHERAPY

The anti leukaemic drugs all damage leukaemic cells but cannot eradicate them The recent observations by French workers on the Jugoslav scientists exposed to lethal doses of ionizing radiations have shown that the bone marrow apparently completely destroyed will recover if the patient can be kept alive long enough during the aplastic period and Mathe s results in the treatment of lymphoblastic leukaemia in childhood by means of lethal doses of ionizing radiation suggest that leukaemic cells will also regenerate months after their apparent destruction It would seem therefore that the aim of conventional chemotherapy should be to suppress the proliferation of the leukaemic cells rather than to destroy them In practice a compromise is struck between the risks inherent in the use of toxic drugs and the benefits that accrue from suppressing the proliferation of the leukaemic cells The compromise breaks down when the treatment harms the patient more than the disease does or when as inevitably happens the disease becomes resistant to the treatment Since the major effects of the very numerous drugs in each of the main classes are so similar the choice of drugs depends mainly on secondary considerations such as chemical stability ease of preparation and marketing convenience of administration freedom from side effects speed of action and the ease with which dose adjustments can be made Familiarity with these factors requires experience and any one worker will obtain the best results with the drugs he knows best

CHRONIC GRANULOCYTIC LEUKAEMIA

It is unusual nowadays to see patients who receive no treatment throughout the course of chronic granulocytic leukaemia but from the few patients who present with advanced disease or who have for one reason or another never received specific treatment we know that untreated patients are severely anaemic invalids with grossly enlarged spleens and livers The leucocyte count is frequently in the neighbourhood of 500 000/mm^3 and occasionally reaches 1 000 000/mm^3 This is the only form of leukaemia in which the leucocyte count provides a reliable indication of the activity of the disease for there is an extremely close relation between the height of the leucocyte count on the one hand and the clinical condition and the severity of the anaemia on the other When the count is made to fall to normal levels by any effective

CHEMOTHERAPY OF LEUKAEMIA

D A G GALTON

Since leukaemia is incurable certain questions arise concerning the aims of treatment in general First does treatment prolong life or merely relieve symptoms? If life is not prolonged is there any justification for treating the symptomless patient? Second what risks does treatment involve? The answers to these questions in so far as they can be given at all, are not the same for all types of leukaemia because of basic differences in their pathology Besides these general considerations chemotherapy poses problems of its own How far is chemotherapy a satisfactory alternative to radiotherapy? Are the two types of treatment competitive or complementary? What considerations influence the choice of drugs and the way in which they are used? Again the answers vary with the type of leukaemia

There are two practical considerations which apply to the chemotherapy of all types of leukaemia The first concerns recording Anti leukaemic therapy causes more or less drastic changes in the numbers of circulating blood cells, which reflect the effects of the treatment on the haemopoietic cells These changes are much more easily followed on a chart than from lists of figures just as temperature readings are and the patterns of response to therapy have the same kind of significance as the well known patterns of temperature charts Most leukaemia therapy is conducted on an out patient basis and it is convenient for patients to have their blood taken on arrival so that at least the haemoglobin, total leucocyte and platelet counts can be charted before the treatment is prescribed, for treatment may have to be changed or stopped not so much because of the absolute levels of the blood counts but rather because of the rate at which they change This is relatively easy to appreciate from the changing slopes of lines on a chart The charts are also important in comparing the response to therapy with previous courses of treatment by the same or different methods and in recognizing a change in the evolution of the disease before it becomes clinically apparent It is useful to indicate all details of therapy on the chart and to note the patient s weight essential clinical data and the dates of bone marrow examinations The second practical consideration concerns the time required to secure the full benefit of therapy This may be six to twelve weeks in acute leukaemia and four to six months in the chronic forms It is therefore important to avoid the temptation to change the treatment too soon or too often Once a drug has been chosen it is equally important to keep to the dose decided on sufficiently long for its effects to

disease higher doses will be required to keep pace with the increasing rate of granulocytic proliferation

Symptomless patients seen for the first time should probably always be treated if their haemoglobin levels are below normal Patients with normal haemoglobin levels may be kept under observation for a number of weeks while the activity of the disease is assessed

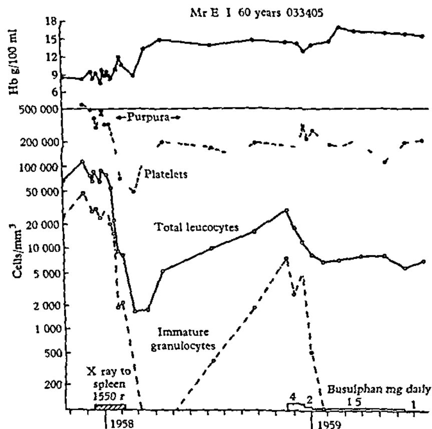


Fig 1 Chronic granulocytic leukaemia Changes in haemoglobin concentration in platelet counts total leucocyte and immature granulocyte counts following radiotherapy and busulphan therapy

Comparison of the value of busulphan and radiotherapy

Busulphan therapy is as effective as radiotherapy in the initial treatment of chronic granulocytic leukaemia and has the advantage of being much easier to administer and more convenient for the patient It is the treatment of choice in patients whose leucocyte counts increase too rapidly for

type of therapy, the spleen shrinks the numbers of erythroblasts increase in the marrow, and the haemoglobin level rises But when treatment is discontinued, the granulocytic proliferation almost always begins immediately, and the leucocyte count increases exponentially For this reason a chart incorporating a semi-logarithmic scale is specially valuable since the exponential increase appears on it as a straight line thus permitting a remarkably accurate prediction of the future trend in the leucocyte count

The pattern is repeated with subsequent courses of therapy, but the rate of increase in the leucocyte count tends to rise, so that the slope made by joining the points representing successive counts rises more steeply After each course of therapy, the haemoglobin level rises but the maximum level reached is never maintained, and the haemoglobin level falls as the leucocyte count increases Intermittent therapy thus gives worthwhile but transient benefit Splenic irradiation is the classical method of applying intermittent therapy and drugs such as busulphan mercaptopurine, or methylcolchicine are often used in the same way Much better results are obtained by giving continuous therapy By keeping the leucocyte count within normal limits, for months or years the haemoglobin is maintained at a high level and the rise and fall inseparable from intermittent therapy is avoided Patients vary in the rate at which their leucocyte counts increase after an initial course of therapy In a few it is so low that continuous therapy is unnecessary In others the rate of increase is so fast that only continuous therapy offers any prospect of providing real benefit Whether or not continuous therapy is required is usually apparent within three months of an initial course of radiotherapy or chemotherapy In the patient whose chart is shown in Fig 1 continuous therapy with busulphan was begun nine months after a course of splenic irradiation The favourable effect on the haemoglobin level is of particular interest and indicates that the effect of the initial therapy was suboptimal This is quite characteristic and results from the very short period in which granulocytic proliferation is suppressed by a short course of treatment

Busulphan is a very convenient drug both for initial and for maintenance therapy in chronic granulocytic leukaemia It may be given more or less continuously for over five years in favourable cases but eventually the disease always becomes resistant to treatment Details of dosage side effects and toxicity have been given elsewhere (Galton, 1959) and it will suffice here to state that for the initial therapy about twelve weeks of treatment at a constant daily dosage is usually required, and that for maintenance therapy the daily dose must be sought by trial and error It will usually be about half the dose used initially Later in the course of the

unusual for the lymphocytic infiltration of the bone marrow to be much reduced Attempts to continue treatment for long periods may occasionally lead to success but the risks of causing serious neutropenia and thrombocytopenia are much increased It is in fact more important to watch the trend of the neutrophil and platelet counts in treating patients with chronic lymphocytic leukaemia than to follow the lymphocyte counts

Local irradiation

It is common for patients suffering from chronic lymphocytic leukaemia but in excellent general health to develop locally troublesome enlargements of lymph nodes or lymphocytic infiltrations of skin or mucous membranes If there is no indication for systemic therapy these manifestations are best treated by local irradiation

Systemic therapy

I shall illustrate the problems of systemic therapy by reference to the use of chlorambucil an orally administered aromatic nitrogen mustard (Everett Roberts & Ross 1953) and of steroids Forty seven patients received up to five courses of chlorambucil and thirty two received steroids

Chlorambucil A total of 104 courses of chlorambucil were given between January 1953 and October 1959 and in fifteen courses steroids were given simultaneously Treatment was given in the first instance only when symptoms had appeared though in retrospect it seems likely that in some cases the symptoms were unrelated to the leukaemia In some cases the main indications for treatment were fall in the haemoglobin or platelet counts or general progression of the manifestations of the disease In the case of the later courses of chlorambucil treatment was often given in the absence of symptoms when it was clear that the disease was again becoming active The details of dosage and of the results obtained will be reported elsewhere but were similar to those already given (Galton Israels Nabarro & Till 1955)

Chlorambucil is well known to be capable of reducing the lymphocyte count and the size of the lymph nodes and spleen but there are conflicting accounts of its effects on the haemoglobin concentration I shall deal here only with this matter and with the toxic effects of chlorambucil In the present series the haemoglobin fell during treatment in nine out of forty two patients during their first course of chlorambucil The fall was transient in five patients whose haemoglobin level subsequently increased to or beyond that recorded before treatment was begun In two instances it was associated with acute infection probably resulting from drug induced

radiotherapy to be practicable Busulphan, however has been thought to induce terminal blastic relapse earlier in the disease than radiotherapy (Blackburn King & Swan 1956 J D N Nabarro personal communication 1959) though this is not accepted by Scott (1959) from his much larger series of patients and is not supported by my own experience A comparative study of the effect of busulphan and radiotherapy is now being made, as part of a therapeutic trial in leukaemia conducted for the Medical Research Council by a working party under the chairmanship of Professor L J Witts

CHRONIC LYMPHOCYTIC LEUKAEMIA

Chronic lymphocytic leukaemia is often thought of as a relatively benign disease frequently discovered accidentally symptomless for many years and commonly occurring in elderly persons whose expectation of life is scarcely affected by it Statistical analysis, however indicates that half of the patients die in less than 2 7 years from the estimated onset of their disease though the remaining patients survive for longer periods and 1 % live for twenty years (Tivey 1954) The clinical manifestations are the direct or indirect consequence of increased lymphocytic proliferation This is responsible for the enlargement of the spleen and the lymph nodes for the progressive infiltration of the bone marrow with ensuing neutropenia and thrombocytopenia and reduction in the effective erythropoietic capacity The red cell life span is often reduced but auto immune haemolytic anaemia occurs only in a minority of patients

Chronic lymphocytic leukaemia is very different from chronic granulocytic leukaemia in its evolution clinical manifestations and response to therapy In many cases the lymphocyte count remains within normal limits throughout the course of the disease although the bone marrow may be heavily infiltrated Patients with low or normal lymphocyte counts may experience great clinical disability whereas other patients with very high lymphocyte counts may remain free of symptoms for years The leucocyte count therefore does not reflect the overall activity of the lymphoid tissue as a whole and does not often serve as a guide to the need for therapy or to dosage In some cases however the lymphocyte count does increase exponentially after each of several courses of treatment but the rate of increase usually remains the same after each course Many drugs as well as ionizing radiations will reduce the lymphocyte count to normal levels and it is unfortunate that many descriptions of new drugs claimed to be of value in chronic lymphocytic leukaemia refer only to this effect The effect on the enlarged lymph nodes and spleen is however variable and it is

unusual for the lymphocytic infiltration of the bone marrow to be much reduced Attempts to continue treatment for long periods may occasionally lead to success but the risks of causing serious neutropenia and thrombo cytopenia are much increased It is in fact more important to watch the trend of the neutrophil and platelet counts in treating patients with chronic lymphocytic leukaemia than to follow the lymphocyte counts

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Chlorambucil A total of 104 courses of chlorambucil were given between January 1953 and October 1959 and in fifteen courses steroids were given simultaneously Treatment was given in the first instance only when symptoms had appeared though in retrospect it seems likely that in some cases the symptoms were unrelated to the leukaemia In some cases the main indications for treatment were fall in the haemoglobin or platelet counts or general progression of the manifestations of the disease In the case of the later courses of chlorambucil treatment was often given in the absence of symptoms when it was clear that the disease was again becoming active The details of dosage and of the results obtained will be reported elsewhere but were similar to those already given (Galton Israels Nabarro & Till 1955)

Chlorambucil is well known to be capable of reducing the lymphocyte count and the size of the lymph nodes and spleen but there are conflicting accounts of its effects on the haemoglobin concentration I shall deal here only with this matter and with the toxic effects of chlorambucil In the present series the haemoglobin fell during treatment in nine out of forty two patients during their first course of chlorambucil The fall was transient in five patients whose haemoglobin level subsequently increased to or beyond that recorded before treatment was begun In two instances it was associated with acute infection probably resulting from drug induced

neutropenia In the six months following treatment the haemoglobin level increased by 1 6–8 4 g/100 ml above the initial level in twenty seven patients including five of the nine whose haemoglobin level fell during treatment Twenty three patients received a second course of chlorambucil, and eleven of these obtained haemoglobin increases of 1 3–9 7 g/100 ml in the six months after treatment Only five of the eighteen patients who received a third course, however obtained an increase in the ensuing six months of 2 g/100 ml or more

The reason for the fall in haemoglobin concentration during therapy is unknown In one patient the red cell survival was almost certainly considerably reduced, since the reticulocyte counts were raised and transfused blood produced no rise in the haemoglobin concentration Chlorambucil is known to depress erythropoiesis in experimental animals (Elson Galton & Till, 1958) and if it were to do so in a patient whose red cell production was greatly increased to balance the increased loss of cells dying prematurely the haemoglobin level would necessarily fall The shortened red cell survival however must in some way be a result of the underlying disease, and the effect of the chlorambucil in reducing the lymphocytic proliferation might conceivably abolish the action of the factor reducing the red cell life span The reduction of the lymphocytic infiltration of the marrow would also increase the reserve capacity of the marrow for erythropoiesis, and the haemoglobin level would increase These speculations are illustrated diagrammatically in Fig 2 An alternative explanation would be that the drug damages circulating red cells and shortens their life span

Chlorambucil does not often cause dangerous depression of the platelet count even when the platelet count is low before treatment In the present series sufficient platelet counts for adequate analysis were performed during and after eighty three courses, in eleven of which one or more platelet counts below 50 000/mm³ were recorded In no instance was a haemorrhagic manifestation of clinical importance associated with these low counts Neutropenia was a much more serious hazard for neutrophil counts less than 1000/mm³ were recorded during treatment and in the eight weeks following in twenty eight of eighty eight courses Serious infection appeared to be a direct result of drug induced neutropenia in seven patients four of whom died Two however were receiving steroid therapy as well as chlorambucil

Steroids Steroid therapy is well known to reduce haemolysis in cases of chronic lymphocytic leukaemia complicated by auto immune haemolytic disease It will also cause the haemoglobin level to increase in patients with anaemia associated with a shortened red cell life span but without demonstrable auto antibodies In these patients the mechanism appears to

be entirely different and is associated with a general effect on the lymphocytic proliferation similar in many respects to that induced by chlorambucil Freyman & Vander (1960) have shown that the red cell life span may be unchanged after steroid therapy and the haemoglobin increase results from increased red cell production The effect of steroid therapy in suppressing the lymphocytic proliferation resembles that of chlorambucil in the marked

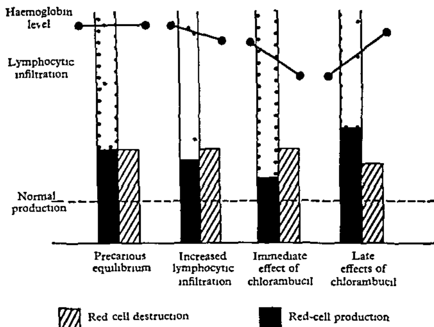


Fig 2 Chronic lymphocytic leukaemia Probable effect of increasing lymphocytic infiltration of the bone marrow and of chlorambucil therapy on red-cell turnover when red cell life span is short Left hand column in each pair volume of marrow normally available for erythropoiesis black columns extent of red-cell production dotted columns volume of marrow not available for erythropoiesis because of lymphocytic infiltration right hand column in each pair extent of red-cell destruction

regression of the enlarged lymph nodes and of the spleen that frequently occurs during therapy but differs in that the lymphocyte count almost always increases in the first eight weeks of treatment instead of decreasing (Fig 3) The effect suggests that the lymphocytes are released rapidly into the circulation from the lymphoid tissues After eight weeks the lymphocyte count begins to fall slowly over the ensuing six months to normal or slightly higher than normal levels The different effect of chlorambucil is almost certainly because of its action in damaging adult lymphocytes as well as suppressing their formation whereas steroids appear only to

suppress the formation of lymphocytes In Fig 3 the haematological response to steroid therapy may be compared with that which followed a previous course of chlorambucil therapy The patient presented with weakness and malaise and was found to have generalized lymphadenopathy

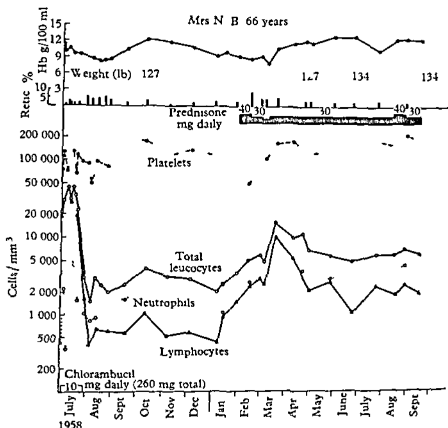


Fig 3 Chronic lymphocytic leukaemia Changes in haemoglobin concentration reticulocytes (per cent) platelet and leucocyte counts following chlorambucil and prednisone therapy

a large upper abdominal mass and a moderately enlarged spleen Following chlorambucil therapy there was almost complete regression of the enlarged lymph nodes including the abdominal mass and the spleen became impalpable The lymphocyte count fell and after a moderate reticulocytosis the haemoglobin level increased When signs of relapse appeared steroid therapy was given

The response to steroid therapy was very similar to the response to chlorambucil, except for the rapid rise and subsequent slow fall in the lymphocyte count Furthermore the remission is being maintained by continuous therapy *Measurement of the red cell life span was not per*

formed in this patient but Freyman and Vander have shown that steroid therapy is effective in causing increase in the haemoglobin level in patients whose red cell life span is normal Figure 4 shows a possible explanation of this which would apply equally well to the effect of chlorambucil When the reserve capacity of the marrow is encroached on by increasing lympho cytic infiltration the haemoglobin level falls The effect of treatment which reduces lymphocytic proliferation is presumably to enable the erythro

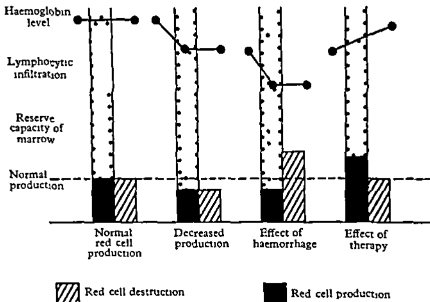


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poietic tissue to respond to the anaemia by producing more red cells The diagram also shows that patients with heavy lymphocytic infiltration who are just able to maintain their haemoglobin at subnormal levels react badly to blood loss They cannot compensate for it and remain anaemic with a haemoglobin level even lower than before

The similarity between the haemoglobin response to chlorambucil and steroids may be further illustrated by comparing the times required before the maximum levels of haemoglobin are attained Thus in six cases in which the haemoglobin increase brought about by steroid therapy exceeded 4 g/100 ml the time for the maximum level to be reached was between

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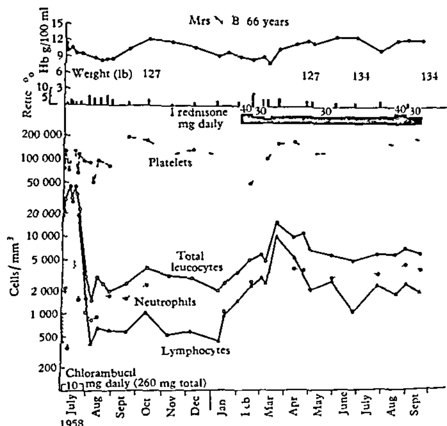


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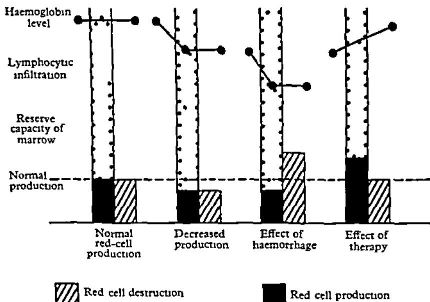


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thirteen and thirty three weeks In nine comparable cases, in which chlorambucil was used, the corresponding time varied between nine and forty weeks

The results of steroid therapy are often dramatic, and steroids may be effective when radiotherapy, chlorambucil and other alkylating agents have failed But there are two special features that render them unsuitable for routine use First, the maintenance of remission requires continuous treatment since relapse almost always follows within three months of dis continuing treatment, and secondly patients who develop even trivial infections during steroid therapy seem unable to localize them and often die from overwhelming infection in a few days Nine out of thirty two patients receiving steroid therapy died in this way, six with pneumonia and one each with septicaemia extensive cellulitis, and pelvic abscess Admittedly most of these patients were gravely ill before steroid therapy was begun and are very unlikely to have survived without it, but it is distressing that in eight cases death from infection occurred less than four months after the start of therapy On the other hand seven patients have taken steroids for twelve to thirty months without mishap The decision to use steroids must therefore always be a calculated risk but the risk is often justified Steroid therapy alone is likely to benefit patients with fulminating haemolytic disease of auto immune type but should otherwise rarely be given as the first line of treatment

Treatment of the symptomless patient

The methods of treatment described are not free from risk, and because of the variability in the speed of evolution of chronic lymphocytic leukaemia and the unreliability of the lymphocyte count in controlling therapy there would seem to be little place for treating symptomless patients as a routine Osgood however has for many years advocated a policy of aggressive therapy in both forms of chronic leukaemia He aims to maintain the leucocyte count below 30 000/mm^3 and does not allow more than three months to elapse without giving treatment It is this policy of virtually con tinuous treatment that he feels to be important rather than the particular method of achieving it So far his long term results are available only for whole body X irradiation and for radioactive phosphorus (Osgood Seaman & Koler 1958) but he is now carrying out a similar programme using several alkylating agents including chlorambucil (personal com munication) Paradoxically it is the patients suffering from chronic lymphocytic leukaemia rather than those with chronic granulocytic leukaemia who appear to have gained most benefit from continuous therapy These patients are reported to have been maintained in better

health and to have survived longer than those treated by conventional methods The greatest benefit was conferred on the patients with the most chronic disease whose expectation of surviving twenty years was increased from 1% to 10% No other worker has yet collected a series of cases comparable with that of Osgood but similar methods are being tried at several centres in the United States

ACUTE LEUKAEMIA

After the initial encouragement that followed the introduction of the folic acid antagonists the steroids and mercaptopurine in the treatment of acute leukaemia the present position can only be described as extremely depressing In adults remissions are disappointingly short rarely lasting longer than a year and there can be no doubt that for the patients who do not remit the effects of treatment are often more distressing than the disease itself In children in over half of whom excellent remissions lasting over a year can be obtained the inevitability of eventual relapse gives one a feeling of hopelessness

To induce remission in acute leukaemia the blast count in the marrow must be reduced to negligible proportions The major clinical manifestations are usually due to neutropenia thrombocytopenia and anaemia resulting from virtual replacement of the bone marrow by blasts When the blasts are eliminated by treatment the marrow is left essentially empty except for fat cells When the therapeutic agent is an antimetabolite it is unusual for regeneration to be effective until the blasts have largely disappeared Until the marrow has regenerated effectively haemorrhage and infection are the main risks to life and the neutrophil and platelet counts if not already lowered by the disease will inevitably be reduced this however does not occur when steroids are used The period of risk varies from patient to patient but it may be three months before the danger is passed

It is often taught that steroids are especially indicated in patients who are acutely ill and in lymphoblastic leukaemia of adults and children mercaptopurine is often said to be especially useful in myeloblastic leukaemia Figure 5 shows the haematological response in a girl of fifteen years with lymphoblastic leukaemia who was treated with mercaptopurine in preference to steroids because she had a salivary gland abscess with cellulitis of the neck She responded twice to mercaptopurine but it is probable that the rapid regeneration of the marrow following the second course was due to the cortisone

Once remission has been induced it is difficult to know whether further therapy should be withheld until relapse occurs or whether maintenance

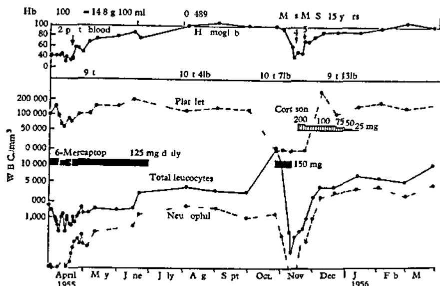


Fig 5 Lymphoblastic leukaemia Changes in haemoglobin concentration platelet total leucocyte and neutrophil counts following two courses of 6 mercaptopurine and one of cortisone therapy

Mrs C age 49 years

Hb 100 % = 14 8 g/100 ml

100 80 60 40 20

Pints blood 3 6 4

125 75 50 75 6 Mercaptopurine

200 000 100 000 50 000 20 000 10 000 5 000 2 000 1 000 500 200

W B C /mm 3

Platelets

Total leucocytes

Neutrophils

Myeloblasts

Aug 1956 Sept Oct Nov Dec Jan 1957 Feb Mar April May

Fig 6 Myeloblastic leukaemia Changes in haemoglobin concentration platelet total leucocyte neutrophil and myeloblast counts following 6 mercaptopurine therapy The daily dosage of 6 mercaptopurine is recorded in mg

therapy should be given in the hope of forestalling relapse Figure 6 shows the haematological response to mercaptopurine therapy in a case of myeloblastic leukaemia Maintenance therapy was not given but the patient remained well for six months Extrapolation of the myeloblast curve suggests that relapse began soon after therapy and that maintenance therapy might have prolonged the remission One of the problems now being investigated in a controlled therapeutic trial under the auspices of the Medical Research Council to which reference has already been made is the relative value of intermittent and continuous mercaptopurine therapy in the treatment of acute leukaemia in adults

I wish to thank the Consultant Staff of the Hammersmith and Royal Marsden Hospitals for permission to quote from the records of patients under their care

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THE POTENTIALITIES OF MARROW TRANSPLANTATION IN TREATMENT OF LEUKAEMIA

H E M KAY

I shall first of all, outline briefly the history to date of bone marrow transplantation, and the attempts to cure leukaemia thereby I shall then examine in more detail some of the critical data on the subject and, finally attempt to show how future experiment and clinical research may be influenced both by our present knowledge and by data likely to be available *within the near future*

The story starts as you are probably aware with the observations of Jacobson (1952) and Lorenz *et al* (1952) on the protective effect against radiation of spleen or limb shielding an effect which was then shown to extend to suspensions of spleen or bone marrow cells injected after radiation For a while there was disagreement as to whether this was due to a humoral factor or to cellular repopulation but it was eventually shown in various ways in many different laboratories that the main if not the sole effect was due to the preservation and subsequent proliferation of intact undamaged cells

Without delay the Harwell group embarked upon the attempt to treat murine leukaemia by large dose whole body irradiation followed by marrow replacement and were soon rewarded by a gratifying measure of success Subsequently as they were quick to point out their results were not nearly so encouraging and with one or two isolated exceptions attempts elsewhere to repeat their experiments failed

Despite this setback there started a series of *clinical adventures in* marrow replacement beginning with a phase of varied but invariably futile endeavours using inadequate X-ray dosage and apparatus but eventually culminating in the careful trials conducted by Mathe and Bernard (1959*a*) in Paris and by Thomas and Ferrebee in Cooperstown (Thomas *et al* 1959)

Now in talking about tissue transplantion I assume you are familiar with the terms I shall use An autologous graft or autograft is tissue removed from and replaced in the same individual an isograft is tissue exchanged between genetically identical individuals a homograft is between unrelated individuals of the same species and a heterograft is between individuals of two different species

Now since most of us are not fortunate enough to possess identical twins let us consider first what it is that determines the fate of tissue homografts Leaving aside such special situations as corneal grafts we can enumerate three distinct factors These are the antigenic differences in the graft host combination the number of immunologically competent cells in the graft and the conditioning of the host

How much do antigenic differences matter? Obviously they matter very much indeed or plastic surgeons would be using skin homografts more frequently and successfully Even after whole body irradiation in the mouse they are absolutely critical since Uphoff and Law (1959) have shown that a single difference at the H 2 gene locus which determines one type of histocompatibility antigen almost invariably leads to rejection of graft or host even with a graft of marrow alone But where there are only minor differences in antigenic genotype compatible combinations can be found Judging by Ferrebee s difficulties with marrow transfer in dogs (Ferrebee *et al* 1958) the situation is very much the same in that species but in the rabbit on the other hand the antigenic barriers seem to be more surmount able and though the data is very incomplete monkey tissues may also be less mutually hostile Man we must hope stands nearer to the rabbit and perhaps the monkey than to the dog and mouse in this affair It is at least plain that we are in serious need of detailed knowledge of the human homo transplantation antigens We can of course make guesses from our extensive knowledge of erythrocyte antigens although I am personally rather doubtful as to whether for example the rhesus complex applies to any other than erythropoietic tissues but I think that with increasingly reliable data on leucocyte antigens and with the studied use of skin homografts as dressings by plastic surgeons we should eventually be able to classify and assign an order of magnitude to the transplantation antigens similar to that which has been established for mice

Figure 1 from the data of Van Bekkum and Vos (1957) shows the results to be expected from isografts homografts and heterografts in mice after varying doses of whole body irradiation The irradiation in this experiment is given to the animal in a short time and the LD 100 of controls is about 600 r You will note that at all dosages up to 1000 r the isograft (CBA) has a protective effect but that in this so called median lethal range the homografts (C57BL) and heterografts (Rat) have if anything an adverse effect This is because the host s immune system is still able to react against the graft which is thereby useless and which may also by its mere presence at a critical time prevent the recovery of the host s own marrow At higher dosages however both the homograft and heterograft assist in increasing the 30-day survival In many cases there mav be partial or complete reversion of the marrow

tissue as the host cells recover although this is infrequent with the higher dosages

Beyond 30 days other effects come into play, especially that of an immune reaction of the grafted cells against the host causing the so called secondary disease which may or may not be fatal The incidence of this in the case of homografts depends upon the existence of host antigens against which the graft can mount an immunological attack Each particular strain combination, therefore exhibits a different incidence and severity It is possible in these circumstances to measure the advantage to be gained by using immunologically immature foetal cells so as to avoid this complication It must be stated straight away that in many, perhaps most cases secondary

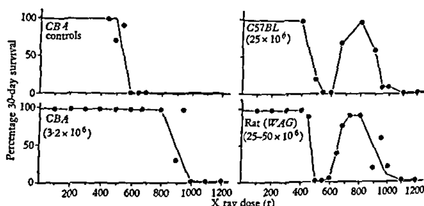


Fig 1 Bone marrow transplantation

disease is not thus avoided although its severity is often less Data from an experiment by Uphoff (1958*a*) showing weight and mortality in control animals in those treated with isologous marrow and in those treated with homologous adult or foetal cells revealed in one instance a clear advantage in using foetal as compared with adult homologous cells

Finally we should take note of the dosage of marrow cells needed to protect irradiated animals This is a topic which is not really understood it appears that for complete protection the dose of homologous or heterologous cells must be about 20–100 times as great as with an isograft

So far I have referred only to grafts of marrow tissue If we consider lymphoid tissue also the results are different First lymphoid cells cannot by themselves protect the animal by repopulating the marrow spaces Secondly isologous lymphoid tissue combined with isologous marrow is at all times and dosages helpful in protection but homologous lymphoid tissue in large doses or isologous lymphoid tissue combined with homologous marrow is worse than useless and leads to increased mortality at all doses

of irradiation It is fairly simple to see why By adding isologous lymphoid tissue to homologous marrow one ensures rejection of the potentially life saving marrow while homologous lymphoid tissue in more than very small amounts will be able to react vigorously against the host to produce fatal secondary disease It is probable that it is the presence of a proportion of lymphoid cells in marrow that causes secondary disease with pure marrow grafts and if this is so the possibility of species strain and individual variation in this characteristic should guard us against assumptions about the degree of antigenicity of tissues in this type of experiment

Thirdly the conditioning of the host must be considered There is scope here for a wide variety of different procedures Irradiation which must be applied to the whole body may be of various qualities may be given slowly quickly or in fractionated doses up to a total dose which can vary by a factor of at least two and of course one must consider the use of adjuvants such as steroids and of alternatives to X rays such as mustards and so forth Altogether it is true to say that the experimental evidence is hopelessly incomplete but with X rays there is enough to suggest that with more prolonged irradiation to a higher dose the damage to the lymphoid system is greater while the damage to the haemopoietic system is perhaps relatively less and to the intestine considerably less The intestine it would seem is an organ that is susceptible more to a particular dose rate than to a total dose Prolonged dosage is obviously therefore an advantage from the point of view of selectively destroying the lymphoid system and so permitting homografts to survive I shall come back to this point again when referring specifically to the treatment of leukaemia

How we may next ask can we translate these procedures from animal experiment to human treatment? The most obvious difference between the laboratory mouse and the hospital man is one of size and this poses a radiotherapeutic problem It is easy with ordinary X ray apparatus to give uniform irradiation at various rates to the whole body of a small animal such as a mouse Not so with man Special apparatus or arrange ments must be devised Dr Mathé uses a Cobalt 60 source in the beam of which a child can lie and receive radiation at the rate of about 50r/hr To achieve uniformity the child is turned every half hour or so and the radia tion has been given on successive nights to a total dose of about 900r Despite sedation nausea and some vomiting is usual and this is aggravated by the necessity of changes of position

The Cooperstown unit has two Cobalt sources 2 m either side of a bed and this is obviously a more satisfactory scheme An even more elaborate arrangement with twelve sources is used at Bethesda to ensure uniformity of irradiation but unfortunately the dose of irradiation is inevitably too

fast In any future scheme care must be taken to enable a wide range of dose rates to be applied down to perhaps as little as 20 or 30r/hr

Irradiation is of course a rather blunt weapon and we must hope in future for chemotherapeutic substances with a rather more selective effect If, however, we are to use marrow homografts, then complete suppression of the immune system is necessary This unfortunately rules out the use by itself at least of such a compound as myleran, and even 6 MP seems to be only immune suppressive in continued dosage at very high levels More lympholytic substances such as chlorambucil, however, are probably as effective as radiation in facilitating the establishment of homografts It is significant that the only successful artificial human chimaera yet achieved followed treatment with amino chlorambucil

Whatever is used the treatment must be drastic and pushed to the limits The patient will then pass through a prolonged phase in which life is threatened by a number of critical events I do not want to go into details about this as you can imagine such a regime requires scrupulous attention to detail In Mathe s cases the list of drugs antibiotics, steroids vitamins nutritional supplements gamma globulin, mouthwashes skin applications and so forth reads like the complete pharmacopoeia Haemorrhage is a constant threat that may be avoided by the use of repeated platelet trans fusions Infection is undoubtedly the most serious risk and one to which most research should be directed The first and obvious precautions are isolation of the patient and reverse barrier nursing by one or two selected pathogen free attendants Our own experience has been that to find proper nurses with sufficient intelligence and skill and without either streptococci or staphylococci in their nose and throat is almost impossible One can attempt to overcome the problem by placing the patient in a sort of plastic balloon of sterile air ultra violet irradiation can be used at discrete points the food must all be sterilized and so forth

However even if all pathogens are excluded from the patient s environ ment there remains the danger from within The normal bacterial and fungal inhabitants of the patient s skin and mucous membranes may by virtue of the decreased general resistance of the patient and perhaps aided by occasional mutations turn from innocent saprophytes to pathogens The exact management of this situation is difficult should one aim to eliminate these organisms before treatment starts or should one wait with antibiotic weapons poised to hit each delinquent organism on the head as it emerges? At present the treatment of the defenceless case the aplastics and the leukaemias is confused by the inadequate protection from outside infection, but with the development of proper aseptic nursing the answer should become clearer

The second great practical problem is that of procurement of adequate quantities of marrow Bearing in mind the number of cells required per gram of body weight in the mouse it can be calculated that for a homograft a dose of the order of 10 to 40 billion cells is needed in a normal adult man There is an assumption here that the proportion of stem cells which are those actually responsible for repopulation is the same in all marrow samples However it is possible that the usual method of marrow aspira tion in man is not as effective in extracting stem cells as the way in which marrow is blown out of the shaft of the femur in the mouse Aside from this it is just possible to aspirate up to 15 billion cells from a normal man with multiple punctures and even perhaps by a single puncture of each posterior iliac spine In the case of foetal tissue it is difficult to obtain enough cells unless one pools the cells from two or more foetuses This perhaps is a permissible procedure but it remains to be proved such

I should say a word about marrow storage Except in the case of foetuses which of course are scarce there is no point in building up banks of marrow cells for homologous grafting There is inevitably destruction of a large number of cells by even the best methods of freezing and it is thus much better to keep one s marrow on the hoof as one author describes it.

In the case of autologous marrow however to which I shall refer later on the destruction of cells by freezing is counterbalanced by the reduced requirement perhaps one twentieth of autologous (or isologous) as com pared with homologous cells

I have described to you some of the problems and hazards that attend the transplantation of marrow in animals and man I now want to elaborate on the subject of leukaemic treatment by inference of killing the leukaemic cells and to see how the two processes are capable of combination

Table 1 summarizes some of the published data on the treatment of murine leukaemia by irradiation and marrow transplantation Not included in this table is the paper delivered to the Third Canadian Cancer Con ference by Loutit (Barnes *et al* 1959) which summarizes their experiments and is perhaps the most valuable of all

The points to be noted in this table are that the majority of these experiments were conducted using transplanted lymphatic leukaemias mostly long established standard laboratory tumours Many such tumours have undoubtedly undergone progression in Foulds s sense of the term that is they have acquired a complete insensitivity to hormones or other body controls they are usually rapidly growing and are able to adapt readily to adverse conditions by the production of variants Radio resistance is therefore a frequent but not universal quality of these leukaemias

Table 1 *Treatment of leukaemia*

Authors	Type of leukaemia	Treatment	Time (n c d)	Type of graft	Result
Barnes *et al* 1956	Transplantable lymphatic	1500 r in 25 hr	1 week	(1) Isologous (2) Homologous	2 /25 survivors (3–5 months) 5/ o survivors (3 5 months) N B 2/18 controls cured
Barnes & Loutit 1957	Some leukaemia	950 r in 22 min	1 week	Isologous and homologous	No long-term surv rs
Trentin 1957	Gardner lymphosarcoma (transplantable)	880 r	(1) am day (2) 4 days (3) 8 days	Isologous	( ) 10/11 survivor 190 days (2) 6/ 4 survivors 190 days (3) 1/12 survivors 190 days
T nt n 1959	M ny transplantable one spontaneous	?	?	?	No survivors
M dd k & Djeras 1958	5 standard transplantable (*800 animal*)	600–800 r	24 hr	Isologous	No survivors
Schwartz 958	Radio-resistant transplantable lymphoma	Maximum tolerated	?	?	No survivors
Cole & Ellis *1958*	Transplantable *myeloid*	700–870 r	16 hr	Homologous (resistant strain)	No survivors (early death without evidence of leukaemia)
Uphoff 1958 *b*	o–12 transplantable	?		?	Few survivors 3–5 months (microscopic recurrence)
de Vries & Vos *1958*	Transplantable lymphosarcoma	800 r	4 days	(1) Isologous + lymphoid cell Isologous without lymphoid cells ( ) Homologous + lymphoid cells Homologous without lymphoid cells	Few 100-day survivors (3/2) No 100-day survivors No 100-day survivors No 100-day survivors
Mathé & Bernard 1959 *b*	Transplantable	850	4 hr	Homologous and isologous	No survivors
Mathé & Bernard 958	Spontaneous Akr é avancé	600– 100 r y and	—	Homologous (some with different H 2) Homologous immunized Homologous embryonic	2/ 200 1 days (same H 2 on adult e foetal)
Simonsen *et al* 1958	DMBA induced	rious 4 hr	—	Isologous spleen	Some very short term success

The treatment whole body irradiation was usually administered rapidly, that is at about 50r/min to a total dose which was lethal to 98% or more of the control animals The time that elapsed between inoculation of the tumour and the irradiation varied in different experiments as did the type of graft used

The critical experiments of Barnes Corp Loutit & Neal (1956) were actually posterior in time to those reported in 1957 and differ mainly in the total dose of irradiation and the rate of administration Both doses are *LD98 for the strain of mouse used These successful results have been* repeated by them with one or two other leukaemias but the original one has now become radio resistant The details of irradiation are evidently of absolutely cardinal importance Notice that there is a higher proportion of survivors with isologous marrow the fatal results in the homologous group being due to secondary disease rather than leukaemia The survival of two controls who were siblings has been ascribed to a possible mutation enabling them to resist the leukaemia immunologically

The Gardner lymphosarcoma used by Trentin (1957) is one of the few established radio sensitive tumours It is evident that the tumour must not be allowed to get going before being irradiated but perhaps prolonged radiation would have been more effective

Homologous transplantation has in many experiments obliterated all sign of the leukaemia the animals dying in these cases of secondary disease In no experiments were homografts found to be superior in their end results to isografts In Uphoff s experiments (1958*b*) the few surviving animals were sacrificed at three to five months and showed microscopic evidence of leukaemia obviously growing rather slowly in the lymph nodes This should generate caution in interpreting the results of other trials

The careful experiments of de Vries & Vos (1958) are of interest in showing the possible value of giving lymphoid tissue as well as bone marrow after irradiation They inferred that the presence of the normal cells may have some suppressive effect at a critical time on the neoplastic cells

Finally two attempts to treat non transplanted leukaemia Simonsen s results are really too short term to be evaluated The treatment schedules and range of donors used by Mathe & Bernard (1958) introduce a tremendous number of variables In brief they had two out of about 200 100 day survivors one after homologous but very closely related adult marrow one after foetal haemopoietic cells at 270 days Both eventually died of leukaemia but it is I think rather more than possible that these were not recurrences but leukaemia arising *de novo* in a strain of high susceptibility This is obviously a very relevant point to consider in relation to human leukaemia

There is a type of experiment now extensively used at Oak Ridge and at the Sloan Kettering Institute in New York to test the susceptibility of a tumour to irradiation or chemotherapy In these experiments an animal with a growing tumour is treated with a very large (in fact fatal) dose of the substance or irradiation and sacrificed soon after Known numbers of tumour cells most of them dead or dying are then injected into susceptible test animals to see if the tumour has in fact been destroyed or to see what proportion of the cells has survived

Figure 2 shows the kind of result one may obtain with irradiation of a hardy transplantable leukaemia The experiment was performed by Hewitt & Wilson (1959) of the Westminster hospital and they have obtained a very convincing straight line relationship between the dose of irradiation and the log of the number of cells surviving This is rather a depressing finding as it seems to indicate that even a dose of 2000r will leave one cell out of 100 000 alive and that to kill 9 999 999 out of 10 million cells we

need a dose of 2700r However, this extrapolation is not necessarily valid and, *further*, this was a fairly tough long established tumour Burchenal (1959) at the Sloan Kettering Institute has conducted trials of this sort with irradiation and with a number of well known anti leukaemic compounds The sterilizing' dose for several different leukaemias varied from 3000 to 12,000r, for nitrogen mustard it was three to five times the LD50 value and for folic acid antagonists more than 100 times the LD50 This is not

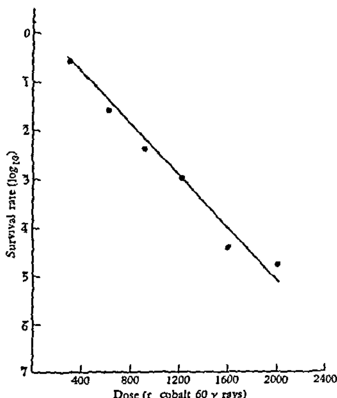


Fig 2 Variation of log survival rate and radiation dose for *CBA* leukaemia cells irradiated *in vivo*

very encouraging information but, of course the same reservations should be made as with Hewitt & Wilson s leukaemia Clearly if it is at all feasible efforts should be made to extend this sort of experiment to spontaneous leukaemias The difficulties admittedly are great

A further expedient would be to combine simultaneously two or more anti leukaemic measures in the hope of achieving a more selective effect This, in fact is how the few human cases have been treated and in this instance experiment seems to have lagged behind clinical practice In normal medical practice of course one keeps as many remedies as

possible up one s sleeve If marrow transplantation became a real possibility a choice would have to be made of the combination of drugs and irradiation to be used in a single major assault upon the leukaemic cells—and also in the case of homografts against the host immune system It might well become necessary to test each particular leukaemia or tumour *in vitro* for its sensitivity to different reagents This is a thorny topic on which conflicting views are held but the work of Schrek (1958) on the radio sensitivity of lymphosarcoma cells shows perhaps how a start could be made

There is another but more dubious way of attacking the leukaemic cells If it is assumed that secondary disease would affect the neoplastic cells more than the normal body cells it could be argued that a mild recoverable form of secondary disease would thus eliminate the few cells remaining from the other modes of treatment Leukaemic animals dying of secondary disease usually show no evidence of residual leukaemia On the other hand there have been even fewer successes in treatment with homologous cells than with isologous cells The symptoms of secondary disease loss of weight with diarrhoea dermatitis and lymphoid atrophy would suggest that it is rapidly dividing cells which are most susceptible They are probably vulnerable by virtue of their cellular antigens It seems to me that the known superiority of neoplastic cells in adaptation and their tendency to simplification and loss of antigens will probably enable them to evade the immune reactions of secondary disea e at least in all cases where the rest of the body recovers I am therefore sceptical of the value of deliberately induced secondary disease in suppressing leukaemia On the other hand as the experiments of de Vries and Vos showed there may well be an advantage in having in the body adequate numbers of normal cells of the same type as the leukaemic cells so that at this critical time there is less stimulus and opportunity for recovery of the damaged but surviving leukaemia cells

At this point it might perhaps be useful to consider one or two actual cases of human leukaemia that have been treated by whole body irradiation The first a case of acute leukaemia described by Mathe had already been through two remissions lasting two years before the necessity for some other treatment arose Irradiation was given on two successive nights followed by two injections of marrow and two more several days later There was rapid depression of the leucocyte and platelet levels requiring frequent platelet transfusions Recovery was delayed until the 22nd day in the case of the white cell count Reticulocytes reappeared in significant numbers only on the 27th day Since there was no increase in the number of foreign red cells at any time after the reappearance of the reticulocytes it seems unlikely that there had been any take of the erythropoietic marrow cells

at least Among incidents during the post irradiation period the most interesting was an episode of gastro intestinal disorder and of dermatitis occurring as long as six to seven weeks after radiation If there had been evidence of graft survival this would have fitted in well with the concept of secondary disease as it occurs in experimental animals Two subsequent cases of Mathe s in whom apparently there was more definite evidence of graft survival did in fact succumb to a similar illness at this phase

A second example is that of an infant with acute leukaemia treated in the Cooperstown unit to a total dose of 950r Numerous injections of foetal cells and some adult marrow were given but in fact when recovery occurred there was no evidence of a take of any of the grafted tissue Relapse occurred within six months as in all the cases of acute leukaemia treated thus far

There are cases where marrow may be used even though a cure is not the aim We have for example, used marrow grafting in a patient with chronic lymphatic leukaemia in whom no conditioning by X rays or chemotherapy had been used immediately prior to injection of foetal cells although previously she had had both whole body irradiation and steroids Hypo plastic anaemia necessitating repeated transfusions and a low platelet count with purpura were present It has been shown that these patients often have an impaired immune system and may tolerate skin grafts for example for longer than normal In this instance we believe there was survival of the graft for perhaps 35 days and it seems that in such cases temporary benefit may be conferred which could quite possibly be repeated

There is another form of marrow replacement which is in current use If during a good remission of leukaemia obtained by 6 MP or other drugs bone marrow is aspirated and stored at $-79°$ C this material is then available at a later stage of the disease when relapse has occurred The relapse may be treated by whole body irradiation or by a large and drastic dose of some anti leukaemic agent followed by replacement with the remission marrow One case has been reported in which a brief final remission was thus gained but in other cases there has been no benefit

I now want to consider the possible use of healthy autologous marrow in the treatment of leukaemia You will have appreciated by now the immense superiority of marrow isografts over homografts in mouse experiments especially to provide supplementary lymphoid tissue and most important of all the fact that one does not need to destroy the host immune system to permit survival of the grafts Consider for example, the possibility of treating myeloid leukaemia with massive doses of myleran Myleran is relatively ineffective against lymphoid cells and so to enable a marrow homograft to take one would be obliged to use also whole body irradiation

or chlorambucil This would be unnecessary if isologous or autologous marrow were available In man isologous marrow is found only in identical twins and possibly existed also in the Egyptian royal dynasties where brother-sister mating was customary Both situations are exceptional but there is no reason why autologous marrow should not be aspirated early in life and kept in store for years as an insurance against radiation accidents or leukaemia Since the dose of autologous cells required is so much less than for homologous cells the deleterious effects of freezing would not be critical and even the aspirative manœuvres would not need to be so very drastic A general anaesthetic is certainly desirable but perhaps the operation could be combined with one of the other rituals of childhood such as tonsillectomy or circumcision There is of course a slight risk as with all operations but if we compare the trouble involved and the risk insured against with say tetanus or poliomyelitis prophylaxis or with vaccination I think you will agree that it is worthy of consideration With a little more data the problem could be submitted to actuarial analysis and perhaps insurance companies could be persuaded to reduce their premiums for marrow banked clients!

There is one big proviso in this scheme and we must return for a few minutes to a consideration of the process of leukaemogenesis I think it is generally agreed that a longish latent period of some years is the rule with most forms of leukaemia extending from its initiation (where this is a single event) to its manifestation During this time a clone or clones of abnormal cells are multiplying in the marrow and probably diffusely throughout the marrow Clearly healthy marrow must be stored before the initiating event and since in the acute leukaemia of childhood such an event quite probably occurs in foetal life during the phase of stem cell proliferation in this instance the proposal is not feasible

In the case of adult leukaemia the marrow laid down in childhood probably would be non leukaemic but there is again a proviso because there might in such persons be an innate predisposition to develop leukaemia They may in other words be like the high leukaemic strain mice who carry an 80–90% chance of developing leukaemia In such a case the leukaemia might well recur in the healthy new grafted marrow This is least likely in cases where external events such as an atomic accident are of greater significance than the innate predisposition But in any case in the process of treating the leukaemia we shall have to render the marrow aplastic with consequent disruption of the delicate mechanisms of haemopoietic homeostasis We should not pitch our hopes too high therefore but I believe that here lies our best chance of achieving a measure of success

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THE HAEMOSTATIC PROCESS

R M HARDISTY

In spite of the ever increasing amount of work devoted to the investigation of various aspects of the haemostatic process we are still far from a complete understanding of the means whereby bleeding is arrested This is largely due to the deceptive ease of planning and carrying out experiments on blood coagulation in glass tubes and the very great difficulty in relating the results of such experiments to clinical observations For example recent work on blood coagulation has revealed the presence of one factor the Hageman factor (HF) whose absence from blood results in a serious deficiency of coagulation in glass tubes but does not impair haemostasis and another factor VII deficiency of which results in a bleeding tendency although blood coagulation can apparently occur normally in its absence

But it is of course obvious that haemostasis depends on other physiological mechanisms besides blood coagulation We know for example that superficial stab wounds as inflicted in performance of the bleeding time test will stop bleeding as quickly in patients with haemophilia as in normal subjects and that women with congenital afibrinogenaemia whose blood is completely incoagulable do not suffer from menorrhagia In thrombocytopenic purpura on the other hand menorrhagia is a common symptom and the bleeding time is usually prolonged Excessive bleeding may also result from a defect of vascular function in the presence of a normal platelet count and blood clotting mechanism examples of this are haemorrhage from an incompletely transected artery in which the normal retraction and vasoconstriction is prevented from occurring and from smaller vessels *in the Henoch–Schonlein syndrome and* von Willebrand s disease

I am sure that these observations will be so familiar to all of you that you will wonder why I bother to mention them I do so simply in order to emphasize that the most direct evidence we possess about the haemostatic process is derived from clinical observations on patients in whom it is defective

In order to study the haemostatic process it is necessary to injure blood vessels and observe the results In man we have only one test of this sort —the bleeding time in which skin capillaries and other small vessels are damaged more or less at random by needle puncture We know that bleeding from such wounds is arrested normally in patients with gross defects of blood coagulation who may yet bleed to death from other types

of injury, even relatively trivial in extent Clearly then different types of injury, involving vessels of different sizes and in different anatomical situations, depend on different mechanisms for the control of bleeding This is borne out by the *striking difference in symptomatology between* various types of bleeding disorders The best test of haemostatic function in man is thus provided by clinical observations of the response to surgical and accidental trauma Experimental results whether on blood coagulation and platelets *in vitro* or on deliberately injured animals may help to define the nature of a particular defect, but can contribute to knowledge of the haemostatic process in man only in so far as they can be correlated with such clinical observations I intend in this lecture to review recent work on the subject in this light—an approach which I am afraid will leave us with a good many unanswered problems

I propose to confine myself to the problem of how bleeding is arrested following injury to vessels I think this will give us quite enough to think about without considering the other aspect of haemostasis—the prevention of leakage of blood from apparently undamaged vessels which clearly concerns the platelets as well as the vessels themselves but not, so far as we know the coagulation mechanism

It is clear from the examples I have quoted and many others that the haemostatic process, in this somewhat restricted sense has at least three *major components—the reaction of blood vessels to injury platelet activity* and the coagulation of blood Each of these three aspects of the problem—especially the last—has been studied separately for many years and there has been much argument about the relative importance of each *but it is* only comparatively recently that the extent of their interdependence has begun to be appreciated I shall begin by considering the three components separately but I shall not be able to keep them apart for long and indeed *it would not be profitable to do so*

VASCULAR FUNCTION

It is common knowledge that large arteries and to a much lesser extent large veins retract and undergo vasoconstriction immediately on being cut and that in the case of arteries this combined with a *fall in blood pressure* may result in the arrest of bleeding before clotting has had time to occur In the case of small vessels and particularly capillaries there is no such general agreement about the importance of vasoconstriction in *the control of haemorrhage*

Macfarlane (1941) observed that normal human nail bed capillaries disappeared from view on injury even when the venous pressure was

raised to 30 mm Hg and reappeared between 20 min and 2 hr later in thrombocytopenia and von Willebrand s disease the capillaries did not disappear but bled continuously He suggested that the disappearance of capillaries was due to their contraction and that this was sufficient to arrest haemorrhage and allow the extravasated blood to clot firmly On redilatation of the capillaries haemostasis could be maintained by the clot

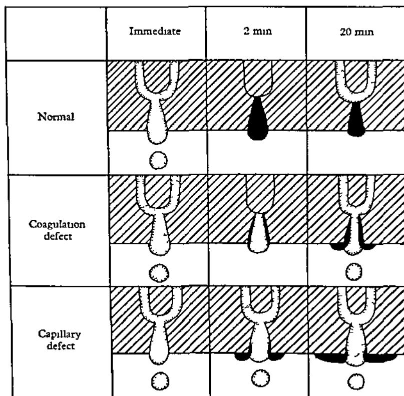


Fig 1 Diagram illustrating the time relationship of capillary contraction and dilatation and blood coagulation The dotted areas represent fluid blood and the black areas blood clot the detached drops indicate active haemorrhage (From Macfarlane 1945 by permission of the editor and publishers of the *Proc R Soc Med*)

already formed or in the case of small punctured wounds by firm adhesion of the wound edges which might make the presence of blood clot unnecessary If clotting was defective bleeding from larger wounds would recur when the vessels relaxed again

This hypothesis represented diagrammatically in Fig 1 offers an explanation of the long bleeding time in the vascular purpuras and von

Willebrand s disease and *of the characteristic delay in the* onset of bleeding after injury in haemophilia and other coagulation defects The main objection to it is that it takes *no account of the platelets except in* their relation to blood clotting, the long bleeding time of thrombocyto penia is attributed to an associated capillary defect

Many other workers have doubted whether capillaries are capable of active vasoconstriction but Chen & Tsai (1948) studying the frog and rabbit have shown that this property varies in the two animals and also from one tissue to another in the same animal So far as human skin capillaries are concerned the work of Sir Thomas Lewis (1927) certainly suggests that they are contractile Chen and Tsai also support the two stage view of haemostasis—that is that the initial vascular response allows the formation of a firm clot which maintains haemostasis when redilatation occurs However while they accept that active vasoconstriction plays the main part in the initial arrest of bleeding from arteries they believe that capillary haemostasis depends more on adhesion of the capillary walls at the site of injury

THE PLATELETS

In 1882 Hayem found that when the jugular vein of a dog was cut, a mass of agglutinated platelets formed so as to fill the opening and apparently *to arrest the bleeding* M B *Zucker (1947) observed the same* phenomenon on transection of mesenteric arteries and veins in the rat and of venules in the rat s mesoappendix, and H D Zucker (1949) in his histological study of small puncture wounds in human skin found that the mouths of all cut vessels larger than capillaries were occluded by platelet plugs H D Zucker saw very few capillaries in his sections and none were occluded by platelet plugs he concluded that capillary bleeding was arrested by fibrin formation but this is inconsistent with the fact that disorders of fibrin formation do not result in a prolongation of the bleeding time

Platelets and vasoconstriction

Although the haemostatic action of platelet plugs may be *partly mechanical* there is much evidence that they may also act by promoting contraction of the adjacent vessels through the *liberation of vasoconstrictor substances* Zucker (1947) noticed that when a muscular vessel was incised in the rat *mesentery it always underwent vasoconstriction in* both normal and thrombocytopenic animals on the other hand vasoconstriction of the adjacent uninjured vessel appeared to depend on the formation of a platelet plug in the cut vessel, and did not occur in thrombocytopenic animals in which no plugs were formed Chen & Tsai (1948) found that the constric

tion of an injured artery persisted much longer when the clot was allowed to remain at the site of injury than if it was washed away

It had been known since the early work of O Connor (1912) Stewart & Zucker (1913) and Janeway Richardson & Park (1918) that serum and platelet extracts contained a powerful vasoconstrictor substance Many later workers confirmed that the serum vasoconstrictor was derived from the platelets (for references see Erspamer (1954)) and the outstanding

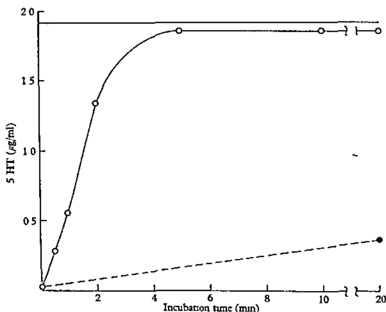


Fig 2 Release of 5 hydroxytryptamine (HT) f om the platelets aft r recalcificat on in glass tubes of the platelet rich plasma of a patient with cong nital afibrinogenaemia ● HT released after incubation of the same platelet rich plasma in glass for 20 min w thout the addition of calcium (From Hardi ty & Pinniger 1956 by permiss on of the ed tor and publishers of the *Brit J Haemat*)

work of Rapport and his colleagues (Rapport Green & Page 1948 Rapport 1949) established its identity as 5 hydroxytryptamine (HT)

It is now known that HT is found in higher concentration in platelets than in any other mammalian tissue Human platelets contain virtually all the HT of the blood and are capable of absorbing HT against a high concentration gradient (Hardisty & Stacey 1955) When blood clots HT is released from the platelets probably in parallel with thrombin formation (Bigelow 1954 Zucker & Borrelli 1955) and certainly independently of fibrin formation as shown in Fig 2 (Hardisty & Pinniger 1956) Now we know that HT is a powerful constrictor of muscular vessels though probably

not of capillaries (Reid, 1943) When we consider these findings together with those of M B Zucker on the rat's mesenteric vessels, to which I have already referred, there certainly appears to be convincing evidence that platelet HT plays an important part in the haemostatic mechanism Unfortunately, however, it is difficult to maintain this view in face of two other observations

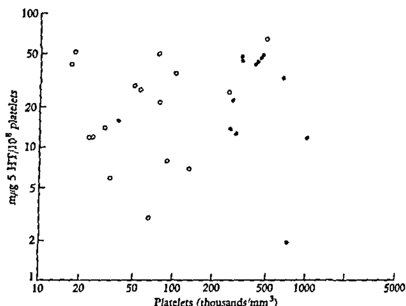


Fig 3 Platelet HT of seventy eight patients with blood disorders plotted against their whole blood platelet counts O patients with purpuric symptoms ● patients with no evidence of bleeding disorder (From Hardisty & Stacey 1957 by permission of the editor and publishers of the *Brit J Haemat*)

First Stacey and I have failed to find any correlation between haemorrhagic symptoms and mean platelet HT levels in a series of seventy eight patients with various blood diseases (Fig 3) the incidence of bleeding manifestations was less closely correlated with the whole blood HT concentration than with the platelet count, as is shown in Fig 4 (Hardisty & Stacey 1957) These findings of course, do not rule out the possibility that HT plays some part in haemostasis but they do suggest that the bleeding of thrombocytopenia cannot be attributed solely to HT deficiency and they provide no evidence that a low blood HT can itself be a cause of abnormal bleeding Similar conclusions have also been reached by Zucker Lewis & Borrelli (1958) who found that serum HT levels correlated poorly with the results of bleeding times and tourniquet tests

Secondly and most strikingly Shore and his colleagues (1956) have shown that gross depletion of platelet HT in rabbits guinea pigs and rats

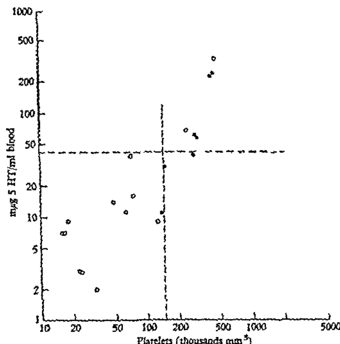
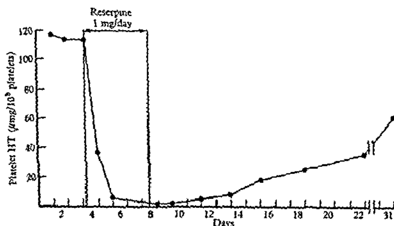


Fig 4 Whole blood HT content of seventy e ght patients with blood disorders plotted against their whole blood platelet counts ○ pat ents with purpuric symptoms ● patients with no evidence of bleeding disorder (From Hardisty & Stacey 1957 by per mission of the ed tor and publishers of the *Brit J Haemat*)



F g 5 Eff ct of 1 mg reserpine pe day subcutaneously on human platelet HT (By permiss on of D R S Stacey)

following the administration of reserpine has no effect on the bleeding time Reserpine has the same action on platelet HT in man (Hardisty Ingram & Stacey, 1956) as seen in Fig 5, but patients receiving reserpine have neither prolonged bleeding times nor haemorrhagic symptoms

Although platelets are evidently capable of binding relatively small amounts of other pharmacologically active amines (Weil Malherbe & Bone 1958, Weissbach Bogdanski & Udenfriend, 1958) there seems little doubt that HT is the substance responsible for the stimulation of vasoconstriction by platelet plugs We are thus left with only two possible conclusions—either that such vasoconstriction forms no part of the haemostatic process at all, or that it forms part of a complex mechanism which can only be dispensed with so long as the other components are not also disordered The first of these is intellectually unsatisfying but the second has some evidence in its favour to which I shall return later

Meanwhile there is another function of platelets to consider—their role in blood coagulation This leads me to a general consideration of the coagulation mechanism

BLOOD COAGULATION

There is no need for me to trace the detailed history of blood clotting theory through the ages I should merely like to remind you that half a century elapsed between the discovery of fibrinogen by Denis (1859) and the formulation of the classical theory of coagulation by Morawitz (1905) as shown in Fig 6 and that the passage of another half century has brought us to the state of our knowledge today, which cannot be expressed more simply than in Fig 7 It has been necessary to postulate the existence of each of the clotting factors represented in this diagram in order to explain the findings in the blood of patients with haemorrhagic diseases and in the case of the Hageman factor even without an abnormal bleeding tendency

When one considers that the only directly observable change in the blood during clotting is the final conversion of fibrinogen to fibrin the extreme complexity of the chain of events leading up to this end result is astonishing Admittedly this apparent complexity may simply reflect the enormous amount of work which has been done in this field in recent years to adapt the dictum of Parkinson (1958) one might say that the apparent complexity of physiological processes in general increases with our knowledge of them Nevertheless it does make one wonder whether fibrin formation is the sole effective end result of the process or whether some of the intermediate products may not themselves play some other role in achieving haemostasis

At least we know that fibrin formation itself is necessary for the maintenance of haemostasis in man as subjects with congenital afibrinogenaemia whose blood coagulation mechanism appears to be entirely normal down to the stage of thrombin formation (Hardisty & Pinniger 1956) but who are incapable of forming fibrin bleed excessively on injury However it has often been observed that such patients suffer less disability

1 Prothrombin + thromboplastin + Ca^{+} → thrombin
2 Thrombin + fibrinogen ——→ fibrin

Fig 6 The classical theory of blood coagulation

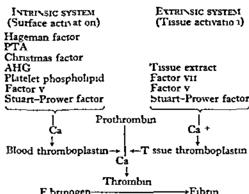


Fig 7 The intrinsic and extrinsic systems of blood coagulation

from bleeding than do many patients with even moderately severe degrees of haemophilia or Christmas disease in whose blood fibrin formation is merely delayed This suggests that the haemostatic defect in haemophilia and Christmas disease depends on more than a simple disorder of fibrin formation and this view is borne out by the fact that the severity of the bleeding tendency in these conditions is more closely correlated with the results of tests of the early stages of coagulation such as the thromboplastin generation test than with the clotting time

The classical theory

But before I speculate any further I had better consider the development of current views of the clotting process in a little more detail According to the classical theory (Fig 6) fibrinogen is converted to fibrin by an enzyme thrombin which is itself formed from a hypothetical inactive precursor prothrombin The conversion of prothrombin to thrombin is brought about in the presence of calcium ions by another hypothetical substance termed

thromboplastin The fluidity of the circulating blood is due to the absence of thromboplastin, which is derived from tissue or platelets following injury

Action of tissue thromboplastin

This theory was fairly generally accepted in its main essentials until the last war but during the 1940 s experiments carried out independently by Quick (1943) Owren (1944, 1947) Fantl & Nance (1946) and Ware & Seegers (1948) led to the conclusion that normal plasma contained a previously unknown substance which accelerated the conversion of prothrombin to thrombin by tissue extract Owren, who studied this substance most thoroughly originally named it factor v, this name is the one commonly used in this country and has now been given official status by the Committee on International Nomenclature of blood clotting factors Factor v is present in fresh plasma but deteriorates progressively on storage of oxalated plasma it is apparently consumed during the course of blood coagulation and is therefore absent from normal serum

The next advance was the discovery that normal serum also contains a substance which accelerates thrombin formation in the presence of tissue extract and calcium The experiments of several groups of workers (Owen & Bollman, 1948 de Vries Alexander & Goldstein 1949 Owren 1951, Koller Loeliger & Duckert, 1951) all lead to this conclusion and the findings of Quick & Stefanini (1949) although originally interpreted differently can be explained in the same terms The agreed international name for this first serum accelerator is the one first used by Koller—factor VII Like most of the other clotting factors now known to be present in normal serum and like prothrombin itself factor VII is adsorbed by inorganic precipitates such as barium sulphate and aluminium hydroxide and is deficient in the blood of patients with vitamin K deficiency or severe liver disease or who are receiving drugs of the dicoumarin group

These two discoveries make it no longer possible to use the term thromboplastin to represent both an active principle of tissue extracts and a substance which is capable of converting prothrombin to thrombin in the presence of calcium ions alone The term is frequently used in the sense of the latter part of this definition but tissue extracts can no longer be said to contain a complete thromboplastin in this sense several workers have shown that they must undergo preliminary reactions with factors v and VII and calcium before an active prothrombin converting principle is formed (Biggs Douglas & Macfarlane 1953c Flynn & Coon 1953 Owren Rapaport Hjort & Aas 1954 Hardisty 1955)

Blood thromboplastin

Neither the classical theory nor these modern extensions of it help to explain the failure of coagulation in haemophilia for haemophilic blood clots normally in the presence of tissue extracts On the other hand normal blood collected without contamination by tissue fluid clots rapidly whereas the clotting of haemophilic blood obtained under the same conditions is much delayed These considerations together with the observation that removal of platelets from plasma greatly delayed coagulation led Quick (1947) and Brinkhous (1947) to the conclusion that thromboplastic activity was developed in shed blood as a result of a reaction between platelets and anti haemophilic globulin (AHG) the plasma factor which Patek & Stetson (1936) and others had previously shown to be lacking in haemophilia

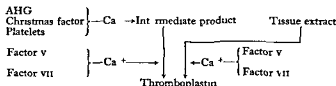


Fig 8 A hypothesis of thromboplastin formation as envisaged in 1953

The concept of intrinsic blood thromboplastin formation was developed and extended by the now classical work of Biggs Douglas & Macfarlane (1953*a* *b*) who showed that the apparent weakness of the thromboplastic activity of shed blood was in reality due to a lag phase during which a series of reactions led up to the eventual explosive appearance of a very powerful thromboplastin These preliminary reactions were shown to involve not only platelets and AHG, but also factor v and one or more serum factors The activity of serum was at first thought to be due to factor vii but with the recognition of Christmas disease as an entity distinct from haemophilia (Aggeler *et al* 1952 Biggs *et al* 1952) it became clear that the Christmas factor (CF) which was present in normal serum was also necessary for blood thromboplastin formation

At this stage a possible representation of the events leading up to thromboplastin formation was that shown in Fig 8 The experiments of Biggs Douglas & Macfarlane (1953*b*) suggested that exposure to glass initiated the process by an action on both platelets and CF As neither platelets AHG nor CF were needed for thromboplastin formation in the presence of tissue extract it appeared reasonable to assume that the active principle in tissues acted in the same way as an intermediate product of these three blood components and was perhaps even identical with it

During the next few years, however, several new discoveries made this view no longer tenable Rosenthal, Dreskin & Rosenthal (1953) described a familial bleeding diathesis due to deficiency of a previously unknown plasma thromboplastin factor, which they called *plasma thromboplastin antecedent* (PTA), and Ratnoff & Colopy (1955) studied three patients with long clotting times but without bleeding symptoms, and showed that their plasma was deficient in another active principle which they named Hageman factor (HF) after their first patient

Meanwhile Hicks (1955) investigated a girl with a bleeding disorder who appeared to have a congenital deficiency of factor VII, and found no abnormality either of thromboplastin generation or of the reaction of the patient s plasma with her *own blood thromboplastin In other words* this patient s clotting defect was only demonstrable in the presence of tissue extracts Ackroyd (1956) and others have subsequently amply confirmed that factor VII plays no part in blood thromboplastin formation but is *required for the coagulant action of tissue extracts It has* since *become* clear that at least one other serum factor is required for the action of tissue extract on plasma, and that it is also concerned in blood thromboplastin generation This factor is now commonly referred to as the Stuart-Prower Factor (SPF) *after the two patients investigated by* Hougie Barrow & Graham (1957) and Telfer Denson & Wright (1956) respectively both of whom suffer from a congenital deficiency of this substance This factor has now been officially designated factor X this implies that it is thought to be identical with the hypothetical serum factor of that name whose existence Koller (1955) postulated in order to explain his findings in thromboplastin generation tests using the serum of patients receiving the dicoumarin group of drugs

Thromboplastin formation and haemostasis

So we arrive at the scheme depicted in Fig 7 which represents two distinct systems of thromboplastin formation intrinsic and extrinsic I must emphasize again that isolated *deficiencies of each of the plasma* factors shown have been observed and that all these deficiencies with the single exception of HF deficiency result in a tendency to bleed excessively on injury Factor V and SPF are required for both systems factor VII for the extrinsic system only and several other factors for the intrinsic system This scheme has been arrived at largely as a result of experiments carried out in glass tubes and it is necessary to ask whether both intrinsic and extrinsic systems are required for haemostasis From the fact that factor VII deficiency results in a bleeding disorder we may infer that the extrinsic system must play a part in the arrest of haemorrhage So far as the intrinsic system is concerned it has been shown that haemo

philic blood will clot normally on the addition of tissue extract even if this has been prepared from a haemophilic subject (Brown 1952) There seems therefore to be no defect of the extrinsic system in haemophilia and it must be assumed that the intrinsic system is also required for haemostasis It thus appears that the arrest of bleeding from vessels in which blood coagulation plays a part depends on the occurrence of both these sets of reactions and that breakdown of either may lead to uncontrolled haemorrhage

The initiation of coagulation

It is not difficult to imagine that the contact of shed blood with tissues initiates the extrinsic thromboplastin system but the trigger mechanism for the intrinsic system is not at all clearly understood It is usually assumed that the initial stimulus is contact with a foreign surface —in the case of most experimental work glass and *in vivo* presumably endothelium which has been altered in some way by injury Many different views of the nature of this foreign surface reaction have been put forward— I have already referred to those of Biggs Douglas & Macfarlane (1953*b*) who thought that both CF and platelets were involved Rapaport Aas & Owren (1954 1955) produced evidence that factor VII was also activated in this way

Following the work of Shafrir & de Vries (1956) and Margolis (1957*a* *b*) it became clear that the activation of plasma by glass depended on the presence of HF Further experiments by Biggs *et al* (1958) Soulier Wartelle & Menache (1958) and Waaler (1959) take us one step further and suggest that the first stage is activation of HF by the glass surface this leads to the activation of PTA and subsequently to the initiation of further stages of thromboplastin formation Platelets and calcium appear to play no part in the early reactions involving contact Hardisty & Margolis (1959) have shown that HF is activated by adsorption on to the glass surface and exerts all its effects in this state PTA subsequently becomes attached to the surface but is only activated if HF is present the activated PTA then probably returns into solution to initiate the later reactions

Now we know that subjects with HF deficiency do not suffer from abnormal bleeding so it appears that these intriguing early reactions which occur when blood or plasma is placed in glass tubes can have no relevance to the problem of haemostasis We must assume that there is an alternative method for the activation of PTA *in vivo* as PTA deficiency does lead to a bleeding tendency Dr Rosemary Biggs has suggested (Biggs 1959) that another clotting factor may be involved and has pointed out that deficiency of such a factor would be difficult if not impossible to detect as it would be expected to produce a bleeding tendency without any disorder of

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coagulation *in vitro* However, it is not absolutely necessary to postulate the existence of another factor to explain this anomaly it may be that PTA is capable of direct activation by damaged vascular endothelium in the absence of HF, but that inorganic surfaces such as glass and silica in its various forms require the presence of HF as an intermediary It is pertinent here to remember that fibrin formation whatever its role in haemostasis also plays a part in other reactions to injury and infection and in foreign body reactions it may be that one function of HF is to initiate the formation of a fibrin barrier round siliceous and other foreign particles It is known that contact of normal plasma with glass leads not only to the initiation of clotting but also to the release of a pain producing substance now usually called plasma kinin, which stimulates smooth muscle and produces vaso dilatation (Armstrong Jepson, Keele & Stewart 1955 1957) and a perme ability factor (Margolis 1958*a*) Margolis (1958*b* 1959) has shown that all these reactions are dependent on the presence of HF As the cardinal features of inflammation include coagulation pain vasodilatation and increased permeability there seems good reason to suppose that HF is concerned with this aspect of coagulation rather than with the haemostatic process

Platelets and blood coagulation

It has been realized since the days of Hayem (1878) and Bizzozero (1882) that platelets played some part in blood coagulation Early workers such as Morawitz (1905) took the view that platelets in shed blood provided a thromboplastic activity similar to that of tissue extracts However we now have plenty of evidence to the contrary for instance normal platelets will not shorten the clotting time of haemophilic blood whereas tissue extracts clot haemophilic and normal blood equally rapidly As I have already mentioned Quick and Brinkhous showed independently in 1947 that AHG and platelets are both required for the production of a clot pro moting activity in blood More recent work besides making it necessary to postulate the existence of all the other plasma thromboplastic factors I have mentioned has confirmed the importance of the platelets in blood thromboplastin formation and has shown that the active principle in platelets concerned in this reaction is a phospholipid probably ethanol amine phosphatide (O Brien 1956) The details of the interaction between platelets and plasma factors, however are still very much an open subject

The development of platelet thromboplastic activity appears to be associated with the physical changes which the platelets undergo when blood comes into contact with a foreign surface Eberth & Schimmelbusch (1886) described these changes as 'viscous metamorphosis and this term is still in general use The platelets appear to become sticky and to

adhere to each other and to glass surfaces forming clumps they swell and extrude pseudopodia and finally undergo partial disintegration releasing small granules which can be sedimented by high speed centrifugation and with which the thromboplastic activity appears to be associated (Bergsagel & Hougie 1956) Some of the appearances are shown in Plate 1 Various authors have thought that these changes are brought about by the action on the platelets either of fibrin (Apitz 1939) or of thrombin (Quick 1951) But Pinniger & Prunty (1946) have shown that viscous metamorphosis occurs normally in the blood of a patient without any fibrinogen and Wright & Minot (1917) who already recognized that the phenomenon was intimately associated with the early stages of coagulation produced the changes by the action on platelets of serum devoid of thrombin More

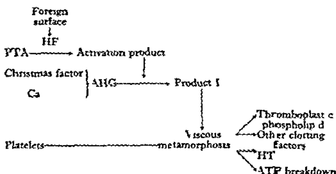
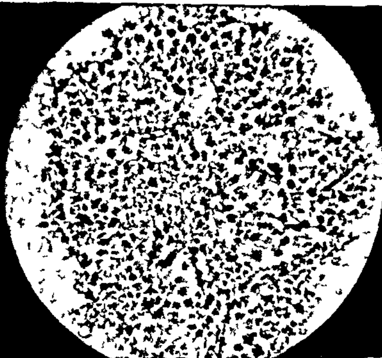


Fig 9 A probable series of changes leading to platelet breakdown during blood coagulation

recently Bergsagel (1956) has produced evidence that viscous metamorphosis is induced by the product of a preliminary reaction between AHG CF and calcium Sharp (1958) on the other hand found that viscous metamorphosis occurred normally in native platelet rich plasma from patients with haemophilia and Christmas disease but was delayed in PTA deficient plasma Biggs and her co workers (1958) found that it was also delayed in HF deficient plasma These findings are difficult to reconcile although they all suggest that viscous metamorphosis depends not only on contact with a foreign surface but also on the occurrence of certain earlier reactions which involve plasma clotting factors only

The experiments of Waaler (1959) on viscous metamorphosis seem to form a link between the findings of Bergsagel and Sharp and the work on plasma reactions to glass contact to which I referred earlier Waaler was able to elute an active clot promoting substance from powdered silica which had been shaken with plasma and he showed that when this

PLATE I



Viscous metamorphosis of platelets in nati e plasma
(From Sharp 1957 by permission)

anticoagulant drugs on the one hand and either thrombocytopenia or variously induced stress on the other (Roskam 1954 Mogenson Fisher & Jaques 1958) Jaques infers that when one of the three lines of defence against haemorrhage—coagulation platelets and vessels—is defective the other two may still be sufficient to maintain haemostasis but that when more than one mechanism is impaired, haemorrhage will result. If we apply this hypothesis to clinical states in man we find that it offers an explanation of the mildness of the disability resulting from an isolated though complete defect of fibrin formation and the complete lack of impairment of the haemostatic process by an isolated deficiency of HT The relatively serious disability of haemophilia and other disorders of the early stages of the coagulation process may be due to the fact that these conditions result in a failure of platelet breakdown which leads in its own turn not only to defective fibrin formation but also to other end results of platelet dysfunction It is curious to reflect that the haemophilic defect was attributed to increased stability of the platelets over thirty years ago (Howell & Cekada 1926) before it was known to be due to the lack of a plasma constituent. We now appear to have come full circle

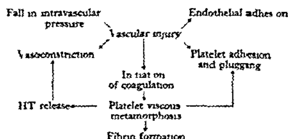


Fig 10 Reactions to vascular injury

A hypothetical scheme of the changes which may occur when a vessel is injured is shown in Fig 10 This demonstrates how the central position of platelet breakdown may form a link between the coagulation process platelet plug formation and vasoconstriction Although the changes of viscous metamorphosis doubtless occur in platelet plugs formed *in vivo* I have not dared to suggest that the initiation of coagulation provides the only stimulus for the formation of such plugs because this has been observed to take place within 2–3 sec after injury when it seems impossible that the clotting reactions could have already occurred (Hugues 1956) This question of the relationship of viscous metamorphosis to platelet plugging of vessels is one which cries out for more investigation a study of the formation of platelet plugs and the release of platelet HT *in various*

activation product' was added to native normal plasma viscous metamorphosis occurred rapidly, even in siliconized tubes AHG, CF and calcium also appeared to be necessary for the rapid occurrence of viscous metamorphosis These findings show that viscous metamorphosis can occur without any direct contact of platelets with glass or other foreign surfaces, and suggest that it is brought about as a result of a sequence of reactions involving HF, PTA AHG CF and calcium, which itself is initiated by contact of plasma with glass A possible representation of such a sequence is shown in Fig 9 which is based on the combined results of Bergsagel & Hougie (1956), Biggs *et al* (1958) Hardisty & Margolis (1959) and Waaler (1959) and is compatible with the findings of many other workers

INTERRELATIONSHIPS OF THE HAEMOSTATIC MECHANISMS

I have dwelt at some length on this problem of viscous metamorphosis and its place in the blood coagulation mechanism because I believe that it may well have more significance than just that of an intermediate stage in the formation of fibrin We know that platelets contain other active principles besides their lipid thromboplastic substance some of these (van Creveld & Paulssen 1951, Deutsch, 1954) are concerned with later stages of the blood coagulation process and others, notably HT, are vasoconstrictor substances Born (1956*a* *b*) has shown that platelets contain fairly large amounts of adenosine triphosphate which is broken down during clotting and may perhaps be an important source of energy for subsequent reactions Little is known about the mechanism of release of HT and other substances from the platelets, but it is tempting to assume that this occurs during viscous metamorphosis, and is therefore dependent on the earlier stages of coagulation proceeding normally Such a hypothesis would be supported by Bigelow s (1954) observation that the release of HT from the platelets was much delayed during the clotting of haemophilic blood as compared with the normal and might also explain the greater severity of the haemostatic defect in haemophilia and Christmas disease than in congenital afibrinogenaemia

You will object that I have already put forward evidence that HT appears to play little part in the haemostatic mechanism but a possible reply to this objection is provided by the experimental results of Jaques (1959) He has found that rabbits and rats treated with either anticoagulant drugs or reserpine (which depletes the platelets of HT) suffer no spontaneous bleeding but when both treatments are combined, spontaneous haemorrhage occurs A similar synergic effect can be demonstrated between

effects of serous exudates produced on injury Our understanding of the haemostatic process will not be complete until we can demonstrate a cause in every case of abnormal bleeding and that we are certainly far from being able to do at present

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disorders of coagulation might shed much light on the whole problem of haemostasis

For similar reasons, vasoconstriction is obviously not solely dependent on release of HT from platelets, large vessels and possibly also some capillaries contract instantaneously on injury, before any platelet change can be observed It may be that damage to capillary endothelium results in electrostatic changes which lead not only to the activation of the blood coagulation mechanism but also directly to retraction of the endothelial cells and aggregation of platelets

Another interesting link between capillary function and blood coagulation is provided by recent work on von Willebrand s disease Until the last few years this was regarded as being due to an inherited abnormality of the capillaries of skin and mucous membranes since these patients have long bleeding times without any obvious abnormality of platelets or the coagulation system It has recently been found however, that many of these patients, including members of the families originally investigated by von Willebrand, have an associated deficiency of AHG (Larrieu & Soulier 1953 Alexander & Goldstein 1953 Nilsson Blombäck Jorpes Blombäck & Johansson 1957) Nilsson and her collaborators (Nilsson Blombäck & von Francken 1957, Nilsson Blombäck & Blombäck 1959) have now produced evidence that the capillary defect in this condition is due to lack of a plasma factor closely resembling AHG but distinct from it They have treated patients with von Willebrand s disease with a plasma fraction containing AHG which not only raises the level of AHG in their blood but also corrects the bleeding time and controls bleeding at operation Furthermore they have shown that a similar fraction prepared from haemophilic blood is equally effective in correcting the bleeding time of von Willebrand s disease Further purification of this fraction of normal plasma appears to abolish the effect on the bleeding time without affecting the AHG content These findings thus introduce a new concept—that normal capillary function depends on the presence of one or more plasma constituents

To sum up I can hardly claim to have presented a coherent picture of the haemostatic process as I said at the beginning of this lecture we are still left with many unanswered problems It does appear, however that the various components of the process may be much more closely integrated one with another than was formerly suspected and that when abnormal bleeding occurs, it often does so because of a combination of defects or other adverse circumstances The relative importance of the different mechanisms represented in Fig 10 varies very much from one type of vessel to another and in different anatomical situations and there may well be other factors which I have not even considered such as the mechanical

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RAPPORT M M (1949) *J Biol Chem* 180 961
RAPPORT M M GREEN A A & PAGE I H (1948) *J Biol Chem* 176 1243
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REID G (1943) *Med J Austral* 2 244
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ROSKAM J (1954) *Arrest of Bleeding* Thomas Springfield Illinois U S A

severity of affection and to avoid asking the patient do you bleed easily or copiously? The man in the street will usually think in terms of the amount of blood which flowed at the time of wounding which of course is related to the extent and nature of the wound the relevant question is how long did such and such a wound continue to bleed even intermittently?

The *family incidence* may also be characteristic In taking the family history it is helpful to draw up the pedigree with the informant and to record his impressions of the relative severity of bleeding in the individuals stated to have been affected This not only helps to prevent omissions but is also a safeguard against relatives being recorded as affected on trivial grounds

Processes disturbing coagulation

I also want to emphasize the principle that clotting may be impaired for one of three reasons either the body may be unable to supply the blood with one or more normal components without which it cannot clot properly or something may be destroying or using up these components more rapidly than the body can replace them or alternatively a substance may be present in the blood which is able to prevent the interaction of the normal com ponents so that clot cannot form Now if one examines blood samples from these three conditions those from the first will show deficiencies which can be corrected by the addition of normal blood those from the second will show deficiencies and given time may also be able to produce similar deficiencies in samples of normal blood with which they are incubated and those from the third will impair the clotting of normal blood immediately on mixing but no more so after incubation Thus if the patient s clotting time in some convenient test is longer than that of the control sample this may either be because the patient s blood is inherently deficient in some clotting factor or because the normal factors are being destroyed or consumed in the body more rapidly than they can be replaced or because the patient s blood contains an anticoagulant substance If now the patient s and the control bloods are mixed together the clotting time of the mixture will be nearly normal in the first case in the second case it will become prolonged on incubation if there is a destructive substance free in the blood stream and in the third case the clotting time will be immediately prolonged usually to a value intermediate between the patient s and the control values Thus it is a fundamental manœuvre in this field to observe in parallel the clotting time of the patient s blood a sample of normal blood and a mixture of the two

The mixing test There is an important rider here If two deficient bloods each giving a long clotting time are mixed and the clotting time of the mixture is normal the original samples may be taken to have had different defects since they have corrected each other If they do not correct each

CONGENITAL AND ACQUIRED DISORDERS OF BLOOD COAGULATION DIAGNOSIS AND TREATMENT

G I C INGRAM

I propose to discuss only those conditions in which a failure of haemostasis occurs through inadequate blood coagulation and I shall not deal with defects of the platelets or of the vasculature

I want to start with two rather general principles

Value of the history

The first point I wish to emphasize is the importance of the history in both diagnosis and management, for while symptoms do not of course distinguish between deficiencies of different clotting factors the history can obviously separate congenital from acquired conditions and the symptoms also provide the best assessment of the severity of a bleeding disease

It is surprising how many people one is asked to see who really have no evidence of a generalized bleeding state They are sent up because they have bled in some unexpected or unexplained manner on perhaps one occasion, although when you go into it they have shown normal haemostasis on many others or they have persistently bled from one site, such as the gastro intestinal tract while they have been submitted to dental extractions and a herniorraphy, say without excessive bleeding With the exception of von Willebrand s disease which follows a fluctuating course, and perhaps congenital factor VII deficiency (Ackroyd 1956), a lifelong bleeding tendency of any severity can reasonably be excluded if the history provides information on several adequate haemostatic challenges to which the patient has responded normally Therefore to prevent false trails being followed as well as to obtain helpful information the patient s history must be detailed and must contain as many variations as possible on two themes the *occurrence* of bleeding and bruising in abnormal sites or following what in normal people would have been insufficient trauma and the *duration* of bleeding after injury whether accidental or surgical Individual occurrences should be inquired for and the events compared in one s mind with an estimate of the expected occurrence or duration of bleeding under similar circumstances in normal people Two practical points are of special value to determine particularly carefully the age and circumstances of the first symptoms of abnormal bleeding in assessing the

disease there may actually be two separable factors missing but since so far it appears that both are always involved they may be considered as constituting a single deficiency (Biggs 1956 Fisch Duckert & Koller 1958) With either of these disorders intrinsic thromboplastin production is impaired so that the thromboplastin generation test will demonstrate an abnormality in both cases

The thromboplastin generation test Perhaps I should just explain the principles of this versatile and important test

It is arranged in two stages and in its standard form the reagents of the first stage assemble all the ingredients of intrinsic thromboplastin but are deficient in prothrombin so that thromboplastin is generated but not thrombin whereas the second stage provides a mixture of prothrombin and fibrinogen in effect as an indicator of the thromboplastin activity The first stage is made up of plasma which has been adsorbed with alumina or barium sulphate providing AHG and factor v serum which replaces all the factors removed from the plasma except prothrombin and platelets or a substitute phospholipid The second stage consists of whole normal plasma The reaction mixture is sampled at intervals on to aliquots of the whole plasma and the clotting times in these tubes reflect the thrombo plastic activity in the first stage reaction at the times of sampling It is convenient to work with citrated plasma so that calcium chloride solution is also added to both stages of the test. The thromboplastin generation test therefore suggests a diagnosis of haemophilia when normal activity is found in the serum component but a defect in the adsorbed plasma but Christmas disease gives the reverse pattern a normal activity in adsorbed plasma but a defect in the serum component The full diagnosis still depends on the mixing test I mentioned just now and Biggs s (1957) assay for AHG is based on comparing the ability of the patient s plasma to correct the defective thromboplastin generation of the plasma of a known haemophilic with the corrective ability of a plasma sample pooled from four or more ostensibly normal persons Christmas factor is more difficult to assay but a qualitative test can be run on the same lines In both these deficiencies the prothrombin time is normal and although the whole blood clotting time is classically prolonged this is only true in the severer cases

Other congenital defects

Besides these two disorders isolated congenital deficiencies of other factors may rarely be met with Factors v vii and Stuart-Prower are all involved in the prothrombin time so that if any of these are lacking the patient s prothrombin time will be longer than the control time (we take a ratio of 1 3 1 of the patient s time to control time as the upper limit of normal

other, but are each corrected by normal blood, they probably have the same defect This mixing test is fundamental in distinguishing between different clotting deficiencies

CONGENITAL CLOTTING ABNORMALITIES

Clotting defects may be congenital or acquired

First, I want to discuss the life long congenital abnormalities So far as I am aware these all fall into the first category of clotting disorders the simple deficiency of one or more essential components

Haemophilia and Christmas disease

Haemophilia and Christmas disease are well known and in this country take first and second place in order of incidence in the group

Genetics Genetically these two disorders are sex linked and recessive characteristically affecting males though transmitted down the generations through females so that the propositus may report symptoms of bleeding in his maternal grandfather his maternal uncles his brothers his mother's sisters sons, his sisters sons his daughters sons and so on while his own sons and their children will escape In Christmas disease and in some haemophilic families, carrier females may also show mild symptoms, so that this feature does not exclude these diagnoses in the propositus

Severity In the affected person the age of presentation is often related to severity Severely affected boys may bleed abnormally at the separation of the cord stump or at circumcision and may show spontaneous bleeding at an early age as for instance from the nose the gut the urinary tract and into joints and may bleed excessively when dropping the milk teeth In milder cases abnormal bleeding will only follow injury so that the disease may present with the first dental extractions or at an accident and spontaneous bleeding may never occur We were once asked to see a man who had bled for so long after prostatectomy that even the urologists thought the blood loss abnormal He turned out to be a haemophilic of moderate severity but he had not mentioned a bleeding tendency before operation because he and others in his family had bled excessively only after dental extractions and they had therefore supposed that they merely had something wrong with the gums

The blood defect Turning to the laboratory aspects we know of course that in both haemophilia and Christmas disease there is an isolated deficiency of a clotting factor In our tautological nomenclature these are known as anti haemophilic globulin (AHG) and Christmas factor respectively (although as a matter of fact there is some evidence that in Christmas

There are also the special problems in haemophilia of dental and surgical operations and of multiple intravenous infusions

This means that in the course of his life a haemophilic will throw up problems in the fields covered by a number of different medical disciplines besides many which we now classify as social You will all know of the Haemophilia Centres which have been set up in the last few years by the Ministry of Health and the Medical Research Council and I am conscious that some of you have accumulated much more experience in running a Haemophilia Centre than I have If I may express an opinion I think that one of the things we obviously ought to do in these centres is to bring together a group of colleagues who are willing to interest themselves in applying their various skills to the haemophilic problems of their specialities and so I thought that I might say a little about how we are trying to do this

I must explain that we do not happen to have a clinical haematology unit so that there are no haematology beds Haemophilic children are therefore under the pediatricians with whom we keep closely in touch and older cases may be under any clinical consultant, except that we have a special arrangement with one physician to take on those who come direct to the Centre One of the dental staff does all our haemophilic work and this has been a particularly valuable association Similarly one physician in the Department of Physical Medicine sees all the affected joints The anaesthetist not only anaesthetizes but also takes charge of all intravenous drips A haemophilic s veins are one of his most precious possessions and we think that it helps to have a skilful person to make careful plans for their best use when drips have to be set up Again we have the help of one of the Lady Almoners who finds a number of problems with which she can assist such as arranging home teachers when schooling is interrupted helping men to find more suitable work finding ground floor accommodation for those who are crippled and so on When the diagnosis is first made we arrange for the patient to be seen by all the relevant members of the group and we let the family doctor know what facilities we can offer and we encourage him to get in touch with us whenever he wants to

Surgery in haemophilia

I want to discuss now the particular problem of dental extractions and surgical operations in haemophilics There are two principles to make good the AHG deficiency by transfusion and to protect the healing wound from trauma which might cause fresh bleeding

Transfusion should be aimed at securing a haemostatic level of AHG in the patient s plasma at the time of operation and sufficiently thereafter to

Ingram & Armitage 1959) Factor v is present in adsorbed normal plasma so that in factor v deficiency the prolonged prothrombin time should be shortened by the addition of adsorbed normal plasma Factors VII and Stuart–Prower are removed by adsorption but are found in serum so that a long prothrombin time due to deficiencies of these factors should be corrected by adding normal serum Furthermore factors v and Stuart-Prower are required in the thromboplastin generation test but not factor VII so that this helps to distinguish between deficiencies of VII and Stuart-Prower Deficiencies of contact factors may also be found In blood from these cases the accelerating effect of a glass surface is less marked than in normal blood but other clotting factors will be normal An isolated prothrombin deficiency, *sensu stricto* is exceedingly rare, but could readily be demonstrated, should it be encountered with Biggs & Douglas s (1953) two stage test This is a most elegant procedure which labours under the misfortune of being so seldom required

Afibrinogenaemia is another rarity though well recognized Here no clot will form although all factors other than fibrinogen are present normally The defect is of course corrected by the addition of purified fibrinogen

Multiple deficiencies

A number of cases of double congenital deficiencies have now been described and the transmission of these disorders is of genetical interest Their existence means that factors present in serum and in adsorbed plasma should always both be tested for along the lines described and that one should not put away the stop watches as soon as one deficiency has been detected

This leads me to a discussion of the management of these disorders and I propose to talk in terms of haemophilia on the understanding that similar principles apply to the other deficiencies too

Management

Haemophilia like diabetes is a life long disease which may be punctuated by critical episodes and attended by various chronic disabilities I make this comparison because haemophilia is sufficiently uncommon for many people to have little idea of the haemophilic life whereas we all have some idea of what it means to be a diabetic and so the analogy may be helpful in visualizing the haemophilic problem In fact of course, the severe haemophilic is the worse off in several respects he will have been affected from early childhood his physical activity will be more restricted and the course of his life more severely interrupted his acute episodes may be more unpleasant and the haemophilic has as yet no maintenance treatment

hydrocortisone into the joint space in any event further intravenous infusions of plasma may be necessary for a day or two

The chronically disorganized joints should no longer be regarded as a hopeless problem If the limitation of movement is due to soft tissue adhesion or contracture patient and gentle physiotherapy over many months may extend the range of movement considerably In flail joints of the lower limb fixation in the optimum position covered by AHG infusions has been found to give a more useful limb and in fixed joints of the upper limb arthroplasty should similarly be feasible from about the age of fifteen years

Other *spontaneous bleeding* usually responds well to plasma infusions

ACQUIRED CLOTTING DISORDERS

I turn now to the acquired clotting disorders Except for one group these all seem to belong to my second and third categories where clotting factors are either destroyed or removed more rapidly than they can be replaced or else are prevented from interacting by the presence of an anticoagulant.

Underlying mechanisms

To begin with I would like to discuss the mechanisms underlying these disorders

Excessive utilization In the first place it is possible that *bona fide* attempts at haemostasis in a large wound may overdraw the account so to speak This is supported by some animal work by Brinkhous & Penick (1955) and in man Fitzgerald & Jackson (1956) estimated the fibrin in the clot expressed with the placenta after a delivery complicated by antepartum haemorrhage and obtained the astonishing figure of 63 g Now the circulating blood at term carries about 10 g of fibrinogen so that this clot represented the clearance of some six blood volumes and in laying down this clot presumably other clotting factors were proportionally involved

Metabolic group Secondly I would like to collect together a rather miscellaneous group of conditions under the heading of metabolic disturbances with at any rate this in common that bleeding seems to be due to multiple deficiencies in clotting factors The obvious entry is the bleeding state following overdose of coumarin and indanedione drugs which through interfering with their synthesis or liberation into the blood produce deficiencies of prothrombin factor VII Christmas factor and Stuart-Prower factor Allied to this is the condition arising from a defective intake or absorption of vitamin K whether from dietary restriction biliary diversion malabsorption due to intestinal disease or from destruction by

enable efficient clots to form To plan this effectively, it is helpful to know the rate of removal of AHG from the plasma This is much faster than one might suppose for while albumen is turned over at about 3% per day (Volwiler, Goldsworthy MacMartin Wood Mackay & Freemont Smith, 1955) AHG disappears at about 80% per day (Biggs 1957)

This explains why it is necessary to give so much fresh plasma (or its equivalent in AHG) so often to support a severe haemophilic in a haemostatic crisis In discussing these transfusions I shall use the term 'AHG to include all AHG containing materials—that is whole fresh plasma and purified AHG s from various sources

Generally speaking the patient s previous personal experience as well as the degree of the AHG deficiency may be taken as a guide to the intensity of replacement treatment which will be needed but the AHG level in the patient s plasma should usually be raised to 20–30% of average normal to cover dental extractions and minor and moderately extensive surgery and this may usually be achieved by infusing a litre of ACD plasma in half to one hour within two to three hours of bleeding the donors, having *spun the blood in a cooled centrifuge if possible*

Thus an infusion should be given immediately before the haemostatic challenge, since it seems easier to prevent abnormal bleeding in this way than to arrest it afterwards It may also be necessary to continue daily transfusion until healing is well under way not only to prevent post operative bleeding but because there is a suggestion that healing is delayed by AHG deficiency

Protection of healing wounds is of course very much ad hoc Before teeth are removed for instance guard plates should be made so that they can be applied immediately after the extractions and left in place for some days The plates will protect the sockets during eating and if bleeding recurs absorbable dressings may be lightly packed beneath them preceded, perhaps by another infusion of AHG If indeed anything has to be done which might disturb a healing wound, the procedure may have to be covered by a dose of AHG *given immediately beforehand* Thus after major surgery the changing of dressings and packs the removal of drains and sutures and physiotherapy should all be timed to follow an infusion

A word about *haemarthrosis* A bad bleed into a joint is completely incapacitating and very painful and the acute stage may last several days The later results particularly after several attacks may also be very disabling leading either to immobility or to a flail joint

The emergency may be met by applying a Robert Jones bandage to the affected joint and by infusing a litre of fresh plasma intravenously Some centres are trying aspiration and perhaps following this by injecting

hydrocortisone into the joint space in any event further intravenous infusions of plasma may be necessary for a day or two

The chronically disorganized joints should no longer be regarded as a hopeless problem If the limitation of movement is due to soft tissue adhesion or contracture patient and gentle physiotherapy over many months may extend the range of movement considerably In flail joints of the lower limb fixation in the optimum position covered by AHG infusions has been found to give a more useful limb and in fixed joints of the upper limb arthroplasty should similarly be feasible from about the age of fifteen years

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the colonic flora, the same clotting factors appear to be involved (Quick Hussey & Collentine, 1952, Douglas, 1958, Spaet & Kropatkin, 1958) and the same applies to haemorrhagic disease of the new born (Aballi, Banus de Lamerens & Rozengvaig 1959*a*, *b*) In kwashiorkor and infantile gastro enteritis Merskey & Hansen (1957) found deficiencies of factor VII and perhaps Stuart-Prower and in some cases also prothrombin deficiency although Christmas factor was not affected Finally in liver disease all these factors may be depressed and also factor V and fibrinogen (Favre Gilly, 1947 Cowling, 1956, Rabiner & Spaet, 1959)

The escape of tissue factors into the blood stream Another group of acquired disorders comprises those conditions in which a serious destruction of clotting factors appears to follow the escape of tissue substances into the blood stream Indeed one or both of two quite separate processes may thus be initiated

One possibility is the activation of the extrinsic clotting system within the circulating blood The clotting factor in tissue reacts with the clotting components in the plasma to form extrinsic thromboplastin this converts prothrombin to thrombin, and this turns the fibrinogen into fibrin The process is usually rather long drawn out, so that fibrin forms diffusely enough to be filtered off gradually in various capillary beds without obstructing the circulation Ultimately the red cells are suspended no longer in plasma but in serum, so that the blood is gravely depleted of all those factors which are consumed during normal clotting and thrombocytopenia often develops as well This is what I understand by the defibrination syndrome (Ingram, Norris & Tanner, 1960)

The other possible result of tissue substances entering the blood stream is the activation of the fibrinolytic system This is a system of plasma factors rather like the clotting system with various precursors activators and inhibitors whose end product is a wide spectrum proteolytic enzyme called *fibrinolysin or plasmin* which attacks a number of protein substrates including several of the clotting factors and also platelets Normally this system is quiescent and its physiological role is obscure, but it can be rapidly activated in various ways Indeed, in some people the threshold seems to be much lower than in others so that some persons will develop fibrinolysin by merely running upstairs in many it will develop before examinations and the like and it can often be demonstrated pre operatively Another way in which fibrinolysin can be activated is as I said by the entry of tissue juice into the blood and indeed a very high level of activity may be produced in this way causing severe damage to the haemostatic mechanism and sometimes fatal bleeding An immense literature has accumulated in this field and among a number of reviews may be men

tioned the classic paper of Macfarlane & Biggs (1948) and recent discussions by Cooper (1959) and by Sherry Fletcher & Alkjaersig (1959)

Tissue substances may enter the blood in a variety of conditions and the development of bleeding may be acute or subacute The acute conditions include concealed antepartum haemorrhage (*abruptio placentae*) septic abortion thoracic surgery and incompatible blood transfusion Placenta is rich in coagulant tissue material (Mills 1921 Seegers & Schneider 1951) and in *abruptio* Schneider (1952) has suggested that the rise in pressure in the retroplacental haematoma squeezes placental substances into the maternal circulation and in thoracic surgery the handling of the lung may have a like result Red cells contain a factor with platelet like activity (Georgatsos Hussey & Quick 1955) and this is presumably liberated when incompatible cells are destroyed

Rate of onset In these conditions a severe bleeding state may develop in the course of an hour or two and the clinical diagnosis of this complication will become rapidly obvious but a similar picture may unfold less dramatically over days or weeks in association with retained dead foetus and with hydatidiform mole where maceration presumably leads to the absorption of liquid products and with carcinomatosis particularly from prostate pancreas and stomach where I suppose invasion of normal tissue or necrosis in the neoplasm may allow tissue factor to enter the blood stream Rarely a bleeding state has arisen from a spontaneous activation of fibrinolysin and has continued for some months (Firkin Reed & Blackburn 1957)

Management

The management of these disorders depends on two things In the first place one must consider the primary condition which has allowed tissue material to enter the blood stream In the acute obstetrical emergencies remission always follows the completion of delivery and in the operative cases the blood gradually returns to normal in the post operative period so that the acute bleeding state is self limiting provided the patient survives the immediate hazards The primary condition should therefore be foreclosed as expeditiously as possible and it seems that, in childbed delivery by the natural route is surprisingly safe in the face of haemostatic defects (Israels Lempert & Gilbertson 1951 Peterson & Larson 1954) and hence probably safer than Caesarean section In carcinomatosis and with the spontaneous fibrinolytic states corticoids may be tried We had a case of carcinomatosis from stomach where prednisone seemed to reduce fibrinolytic activity (Fig 1) In carcinoma of prostate the bleeding tendency is said to have lessened under oestrogen treatment (Bergen & Schilling 1958)

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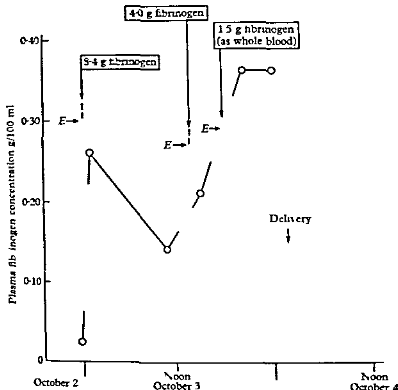


Fig 2 Defibrination complicating *abruptio placentae* A primigravida aet. 27 years was admitted at night in the 34th week of pregnancy with a diagnosis of concealed accidental haemorrhage

At 11 45 p.m. on 2 October her plasma fibrogen concentration was found to be 0·025 g/100 ml. An infusion of 8 4 g of fibrinogen was given following which the plasma concentration rose to 0 26 g/100 ml whereas a concentration of 0·30 g/100 ml had been anticipated from the estimate of blood volume derived from body weight and from the venous haematocrit.

By 11·00 a.m on 3 October the plasma fibrinogen concentration had fallen to 0·14 g/100 ml a further infusion of 4·0 g of fibrinogen raised it to 0·21 g/100 ml, whereas 0·27 g/100 ml had been anticipated. The failure to reach the expected level on both occasions as well as the rapid disappearance of the first infusion suggested a continuance of the defibrination process

At 6 00 p.m. a further 1 5 g of fibrinogen was given as whole blood and by 9 30 p.m the plasma concentration had risen to 0·36 g/100 ml some 125 [illegible] this was taken to mean that the defibrination process was now abating and it was suggested to the obstetrician in charge of the case that natural delivery should be awaited and that a contemplated Caesarean section should not be undertaken because the platelet count had now fallen to 35 000 mm and there was a presumptive evidence (thromboplastin generation screening test) of some degree of AHG deficiency [illegible] would have made operation hazardous

At 12·05 a.m on 4 October the plasma fibrinogen concentration was [illegible] found to be 0·36 g/100 ml although no more fibrinogen had been given [illegible] delivery followed at 1 50 a.m. with minimal blood loss.

E = expected plasma fibrinogen concentration [illegible] (From [illegible] rris & Tanner 1960)

Secondly, from the haematological point of view management turns on which clotting factors have been most reduced If the concentration of fibrinogen has fallen below 0 05–0 10 g/100 ml plasma, and especially if serial estimations show a progressive reduction an infusion of about 5–10 g of purified fibrinogen may be given The fate of the infused material

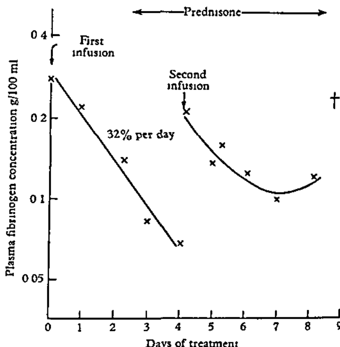


Fig 1 Fibrinolysis with carcinomatosis ventriculi A man of 49 years had undergone partial gastrectomy two years previously for peptic ulceration which was found histologically to be malignant He now complained of two weeks haematuria purpura and superficial bruising Active fibrinolysis was demonstrated in his plasma and the plasma fibrinogen concentration (Ingram 1952) was 0 08 g/100 ml plasma Two doses of 7 6 g of human fibrinogen (Ingram Pinniger & Vallet 1960) were given as shown and their fate in the body followed by making the serial estimates of plasma fibrinogen concentration which are plotted in this figure Prednisone 80 mg daily falling to 30 mg daily was commenced 36 hr before the second infusion and appears to have lessened the rate of disappearance of this dose of fibrinogen Unfortunately the patient died of a cerebral haemorrhage probably related to the associated thrombocytopenia (50 000/mm^3) on the 10th day of observation

The fibrinogen concentrations are plotted logarithmically

then provides a sensitive indication of the continuance of the bleeding process (Figs 1 and 2) If there is a serious reduction in platelets or anti haemophilic globulin it may be possible to replace these by transfusion of the appropriate blood products or simply by large volumes of platelet rich citrated plasma Also in the acute cases an attempt should be made to distinguish between defibrination and fibrinolysis since there is some hope now of the development of specific anti fibrinolytic treatment

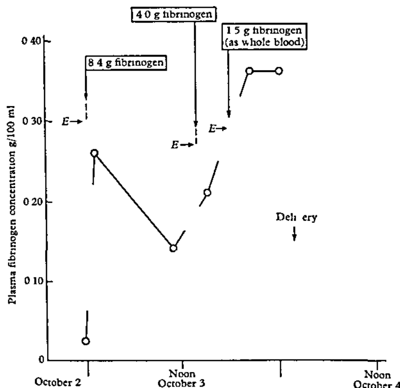


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E = expect d plasma fibrinogen concentrat n following infusions (From Ingram Norris & Tanner 1960)

Secondly from the haematological point of view management turns on which clotting factors have been most reduced If the concentration of fibrinogen has fallen below 0 05–0 10 g/100 ml plasma and especially if serial estimations show a progressive reduction, an infusion of about 5–10 g of purified fibrinogen may be given The fate of the infused material

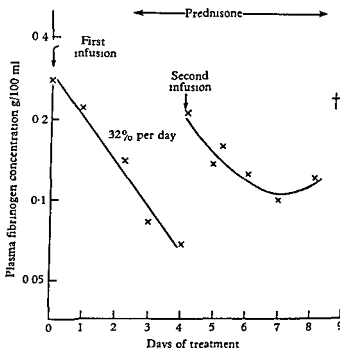


Fig 1 Fibrinolysis with carcinomatosis ventriculi A man of 49 years had undergone partial gastrectomy two years previously for peptic ulceration which was found histologically to be malignant He now complained of two weeks haematuria purpura and superficial bruising Active fibrinolysis was demonstrated in his plasma and the plasma fibrinogen concentration (Ingram 1952) was 0 08 g/100 ml plasma Two doses of 7 6 g of human fibrinogen (Ingram Pinniger & Vallet 1960) were given as shown and their fate in the body followed by making the serial estimates of plasma fibrinogen concentration which are plotted in this figure Prednisone 80 mg daily falling to 30 mg daily was commenced 36 hr before the second infusion and appears to have lessened the rate of disappearance of this dose of fibrinogen Unfortunately the patient died of a cerebral haemorrhage probably related to the associated thrombocytopenia (50 000/mm³) on the 10th day of observation

The fibrinogen concentrations are plotted logarithmically

then provides a sensitive indication of the continuance of the bleeding process (Figs 1 and 2) If there is a serious reduction in platelets or anti haemophilic globulin it may be possible to replace these by transfusion of the appropriate blood products or simply by large volumes of platelet rich citrated plasma Also in the acute cases an attempt should be made to distinguish between defibrination and fibrinolysis since there is some hope now of the development of specific anti fibrinolytic treatment

old age These anti coagulants are fully discussed by Biggs & Macfarlane (1957)

Having described these different mechanisms whereby bleeding states may be acquired in adult life one can now approach the problem of their investigation

Investigation

Test for heparin It is clear that if heparin is present all clotting tests will be more or less affected so that the first step is to exclude the presence of an anti coagulant of this type The simplest thing to do is the thrombin clotting time in which a calcified solution of thrombin is used to obtain a clotting time from the patient s and from control citrated plasmas in parallel

Test for fibrinolysin The next step is to set up a test for active fibrinolysin It is very convenient to use the thrombin clotting time again here along the lines suggested by Wilhelm Miles & Mackay (1955) Samples of citrated plasma from the patient and the control are separately put to incubate Aliquots are removed at intervals and their thrombin times determined Normally of course much the same value should be obtained at each successive determination but if there is a fibrinolytic activity in the patient s plasma the fibrinogen will be progressively destroyed and so the thrombin clotting time will lengthen in the patient s sample

Combined test It will be immediately apparent that in practice these two tests can be run together and in fact if thrombin times are obtained immedi ately the plasma samples are put to incubate these initial readings will serve for the heparin test If then the patient s initial thrombin clotting time is prolonged the presence of an inhibitor can be confirmed by testing mixtures of the patient s and the control plasma in various proportions The test should then be repeated after adding toluidine blue to both the patient s and the control plasmas Apart from the inhibitory mechanism being studied by Kowalski and others to which I have referred if toluidine blue corrects the patient s prolonged thrombin time it may be taken to indicate the presence of heparin If on the other hand the patient s time is prolonged but this is corrected by mixing with normal plasma the prolongation may be due to hypofibrinogenaemia A prolongation of the thrombin clotting time from this cause will be corrected by the addition of purified fibrinogen

If however the initial thrombin clotting time is the same in the patient s and the control sample we leave the plasma samples incubating and proceed to other tests

Tests for deficiencies The next step is to find out what deficiencies exist It would clearly be impracticable to apply routinely a separate test for each

In addition, Kowalski (Niewiarowski, Kowalski & Stachurska, 1959) von Kaulla (von Kaulla & Swan 1958) and others have been working on an inhibitor released from fibrinogen when it is destroyed by fibrinolysin This is still obscure but the hope that it may be elucidated provides an additional reason for identifying the fibrinolytic state

These are not the only processes whereby bleeding may arise, and I have time to discuss one or two more

You will know that heparin is believed to be stored in the metachromatic granules of mast cells, from which it may be liberated into the blood stream I believe that the only physiological circumstance in which this occurs to any extent is during hibernation in those mammals which spend the winter so much more sensibly than we do (Smith Lewis & Svihla 1954) Our blood ordinarily contains a little combined heparin and practically none in the free state but a massive heparinaemia can occur pathologically rendering the blood completely incoagulable This has been described in amniotic fluid embolism complicating tumultuous labour (Ratnoff & Vosburgh, 1952) and in other obstetrical emergencies (reviewed by Ingram Norris & Tanner, 1960) and it has been shown experimentally that an infusion of meconium will produce a like effect (Schneider 1953, 1955) Women suffering from amniotic infusion are said to show extreme vasomotor collapse and this is reminiscent of the experimental syndrome known as 'peptone shock , in which heparinaemia is also characteristic (Howell 1925) Heparinaemia has also been seen as a spontaneous development in otherwise healthy persons (Speer Hill Maloney & Roberts 1955)

An entirely different type of anti coagulant arises in certain chronic disorders Disseminated lupus erythematosus is an interesting example for the inhibitor appears to interfere with the liberation of thrombin (Conley & Hartman 1952 Lee & Sanders, 1955 Bonnin Cohen & Hicks 1956 Ramot & Singer, 1956)

In other circumstances a specific destructive substance appears in the blood The classical occurrence is in haemophilics who have received multiple blood transfusions If a haemophilic has no AHG of his own he may logically regard infused AHG as a foreign protein and no one may complain if he forms an antibody against it This has been described in a few cases and there is one instance on record of an afibrinogenaemic boy who formed a similar anti fibrinogen (Bronnimann 1954) However people who are not haemophilics sometimes also form anti AHG anti bodies which are presumably analogous to auto antibodies formed against other body tissues This has been described in a number of cases following childbirth—a condition quite clearly different from the other obstetrical bleeding states already mentioned and it has also been found in extreme

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clotting factor and so we have worked out the following scheme to obtain as much information from as little work as possible We do a platelet count, since thrombocytopenia is characteristic of both defibrination and fibrinolysis We have a rapid laboratory method for the estimation of fibrinogen in which the clot is weighed after 10 min dehydration in acetone We do Quick's prothrombin time as a combined test for factors v vii and Stuart-Prower (as described earlier on) We use Hicks & Pitney s (1957) thrombo plastin generation screening test for an indication of the activity of AHG and Christmas factor and to show up an inhibitor of thromboplastin generation This of course involves testing a mixture of patient s and control plasmas as well as the two separately so that it is convenient to run the three samples in a staggered concurrent design subsampling from each at 3 and 6 min only Progressive destruction of a thromboplastic factor can be demonstrated by repeating this manœuvre on incubated samples along the lines suggested in the fibrinolysin test If an abnormality is shown, a specific deficiency or the site of action of an inhibitor can be worked out with the conventional thromboplastin generation test

The details of these tests have been described elsewhere (Ingram, 1960) and only a résume will be added here

Plan of investigation Do a platelet count Make 10–15 ml of citrated blood Set up the haematocrit of the citrated blood (this is needed for the calculation in the fibrinogen estimation) and spin the remainder to obtain citrated plasma Similarly obtain normal citrated plasma Begin by putting about 1 ml of each plasma to incubate separately and immediately record the thrombin clotting time in an aliquot of each If the patient s time is prolonged test the corrective ability of an equal volume of normal plasma of purified fibrinogen (for hypofibrinogenaemia) or of toluidine blue (for heparin) If heparin can be excluded leave these plasmas incubating and set up the fibrinogen determination While this is clotting do another thrombin time on the incubated plasmas and do the prothrombin time Now harvest the clot from the fibrinogen determination and transfer to acetone for 10 min Prepare the thromboplastin generation screening test and perhaps do another thrombin time Decant the acetone off the clot evaporate the residual solvent and rapidly weigh the clot Do a thromboplastin generation screening test and set up any further thrombo plastin generation tests which may be indicated Repeat the thrombin time on the incubated plasmas It now should be possible to obtain a fair overall estimate of the abnormalities present and a reasonable approach to replacement treatment can be worked out If deficiencies are found which it would be reasonable to try and correct by transfusions, the specific determinations can be repeated after the material has been given and an

assessment of the rate of progression of the underlying clotting disorder can be made From this helpful information can be offered to the clinician in charge as for instance on the advisability of intervening or of letting the case take a natural course or of the hazards of operating now rather than later

SUMMARY

In summary I will outline the main principles again

First clotting disorders may be either congenital or acquired The congenital disorders appear to be deficiencies of one or more clotting factors and the acquired disorders are probably either multiple deficiencies or are due to some process destroying clotting components more rapidly than they can be replaced or to an anti coagulant substance preventing their interaction

Secondly in the congenital disorders it is now often possible to make a precise haematological diagnosis and to cover haemostatic emergencies by appropriate transfusions In the face of a haemostatic challenge not only must the level of the missing factor be sufficiently restored in the patient s plasma but the wound must be protected during the healing stage from anything which could cause bleeding to recur

Thirdly in the acquired clotting disorders an attempt should be made to distinguish three types of condition the *defibrination syndrome* in which a more or less slow process of clotting occurs in the circulating blood an *activation of fibrinolysin* by which clotting factors are destroyed as they circulate and the presence of an abnormal *inhibitor* or anti coagulant substance These latter may be heparinoid or they may resemble auto antibodies directed against one particular clotting factor or they may inhibit a particular clotting reaction

Lastly the treatment of the acquired disorders is partly to replace those factors which have been destroyed to support the patient in the immediate crisis and also to do what may be done to deal with the underlying process which determined the development of a bleeding tendency

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THE PATHOGENESIS OF PURPURA

J F ACKROYD

Bleeding which is apparently spontaneous or which occurs excessively in response to trauma is of two types it may occur as large extravasations of blood or as small localized haemorrhages which are often only pin point in size and initially bright red in colour and which when seen in the skin form the petechiae which constitute the characteristic clinical picture of purpura The former tend to occur in conditions in which blood clotting is prolonged The latter occur in conditions in which there is primarily a capillary defect the petechiae being due to minute leaks from the capillaries In some conditions in which blood coagulation is greatly delayed as in congenital deficiency of the Hageman factor (Ratnoff & Colopy 1955) or in patients receiving large doses of heparin spontaneous bleeding either does not occur or occurs only very rarely In consequence it seems clear that impaired blood coagulation alone may not be sufficient to cause spontaneous bleeding and that there must also be a vascular defect before bleeding can occur It is not surprising therefore that the dividing line between the two types of bleeding is by no means clear cut Thus extensive haemorrhage —for example fatal cerebral haemorrhage—is not uncommon in purpura Furthermore in some conditions such as for instance von Willebrand s disease which are believed to be due primarily to a capillary lesion petechiae are relatively rare Nevertheless the majority of cases can be divided into these two groups

I propose in this lecture to discuss the pathological changes underlying bleeding of the capillary type and then to consider in detail the patho genesis of several different types of purpura

The vascular defect in purpura can be demonstrated by two simple clinical tests

First the bleeding time is prolonged Although various modifications have been introduced since it was originally described by Duke (1910) this test consists essentially of measuring the time bleeding persists after the infliction with a cutting needle of a small stab wound through the skin I shall be considering later the reason why a prolonged bleeding time is thought to be a manifestation of a capillary disorder

Second increased capillary fragility can be demonstrated by one of the capillary fragility tests of which the positive pressure method of Hess (1916) is probably the simplest and most satisfactory The technique of this test is worth consideration A sphygmomanometer cuff is applied to the arm

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above the elbow and is inflated to a definite pressure for a definite time The pressure to which the capillaries are subjected in this test has been investigated by Landis (1930) who showed that when the cuff is inflated the intracapillary pressure rapidly rises to a level just above the cuff pressure If, therefore the test is performed in the usual way in which it is described in text books, by inflating the cuff to a point 'mid way between the systolic and diastolic blood pressures' (Whitby & Britton 1957), or slightly above the diastolic pressure' (Wintrobe, 1956) the capillaries will be subjected to a wide range of different pressures depending only on the patient's arterial pressure It is clear therefore that some standard pressure must be used for all patients, irrespective of their blood pressures In adults I personally use a pressure of 80 mm of mercury for 5 min In the majority of normal individuals this will produce not more than 10 petechiae in the arm below the cuff

COAGULATION DEFECTS ASSOCIATED WITH BLEEDING OF THE CAPILLARY TYPE

Apart from the frequent occurrence of thrombocytopenia in cases of purpura, the blood coagulation factors are usually normal The following are examples—all of them uncommon—of the association of coagulation defects with bleeding of the capillary type

Von Willebrand's disease Haemorrhage in this condition in which the bleeding time is characteristically prolonged has in the past been thought to be due solely to a capillary defect Recently a deficiency of antihaemophilic globulin has been reported in some cases (Biggs & Macfarlane 1958)

Thromboasthenia In this condition also the bleeding time is prolonged but the capillary defect is associated with a functional abnormality of the platelets which are sometimes also morphologically abnormal The functional abnormality of the platelets can be demonstrated by the thromboplastin generation test (Biggs & Douglas 1953) If in this test platelets from a patient with thromboasthenia are incubated with normal serum and normal aluminium hydroxide adsorbed plasma much less thromboplastin is generated than if platelets from a normal individual are used (Biggs & Douglas, 1953) This subject has been extensively reviewed by Braunsteiner (1955) Thromboasthenia may occur as a hereditary disorder (Glanzmann's disease) A similar platelet abnormality has also been described in haemorrhagic thrombocythaemia (Hardisty & Wolff 1955) Recently Çetingil and his colleagues (1958) have described comparable findings in a case of scurvy—a condition in which the bleeding has

hitherto been considered to be due solely to a capillary defect In this case the platelets reverted to normal after treatment with ascorbic acid

Factor VII deficiency Severe dependent purpura of life long duration has recently been described in a middle aged man with congenital factor VII deficiency (Ackroyd 1956)

The dysproteinaemias The abnormal bleeding sometimes seen in these conditions is due to a combination of factors including capillary defects and abnormalities of blood coagulation (see p 241)

THE ASSOCIATION OF PURPURA WITH THROMBOCYTOPENIA

Although the association of bleeding of the capillary type with deficiencies of plasma clotting factors is uncommon purpura is frequently associated with a reduction in the number of circulating platelets This association raises two problems first why there should be this association and second how far the thrombocytopenia increases the bleeding tendency

It is well known that severe purpura can occur without thrombocytopenia and also that severe thrombocytopenia can occur without purpura This latter point was clearly demonstrated by Dyke & Stewart (1931) who found that in cases of pernicious anaemia the platelet count could be as low as 17 400/mm³ in the absence of abnormal bleeding However the marked reduction in bleeding which has been reported by many workers when the platelet count in patients with idiopathic thrombocytopenic purpura is raised by transfusion strongly suggests that platelets are important in haemostasis The answer to this paradox seems to be supplied by a series of experiments performed by Bedson He first showed (Bedson 1922) that if the capillaries of an animal are damaged to an extent that does not cause bleeding the simultaneous induction of a degree of thrombocytopenia itself inadequate to cause bleeding will cause haemorrhages to develop In a later series of experiments Bedson (1924) found that if the dose of anti platelet serum administered to rabbits was graded so that only the platelets were destroyed then haemorrhages did not develop but that bleeding did occur if a larger dose of the serum was given He was able to show that this larger dose not only destroyed the animal s platelets but also attacked the capillary endothelium

It can be concluded from Bedson s earlier experiments that platelets tend to prevent bleeding from damaged capillaries Danielli s (1940) work would suggest that they do this by blocking defects in the walls of the capillaries Conversely it may also be concluded from Bedson s work that thrombocytopenia tends to increase bleeding due to capillary damage

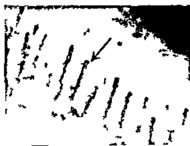
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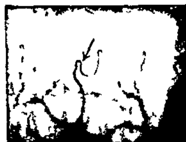
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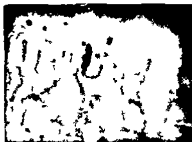




A B

Normal na l fold cap llaries A The arrow ind cates where a c pill ry loop is bout to be punctured B The arrow indicates the site at which the capillary has been punctured Although bleeding has not occurred the capill ry ha undergone contract on and can no longer be seen (Reproduced from Macfarlane (1941) by permi s on of *The Q a terly Jou nal f Med cine*)





C D

Na l fold capillaries in a case of non thromb cytopenic purpura C The loops are rr gul r The rrow indicates where a loop is about to be punctured D Haemorrhage s occurr ng f om the injured vessel which has not contra ted (Reproduced from Macfarlan (941) by p miss n of *The Quarterly Jo r al f Med cine*)

Bedson's later experiments with anti platelet serum suggest that since they are both attacked by the same antiserum, platelets and capillary endothelium are probably closely related antigenically It seems possible that this is the explanation of the frequent association of purpura with thrombocytopenia for, if they are immunologically related, a factor which can damage the endothelial cells may well also damage the platelets and so cause thrombocytopenia

THE CAPILLARIES IN PURPURA

The mechanism of the formation of petechiae has been studied by Humble (1949) Using a microscope arranged for investigating the capillaries in the skin, he observed the formation of petechiae during the performance of the Hess capillary fragility test Petechial haemorrhages developed only at the arteriolar ends of the capillary loops

Macfarlane (1941) has observed the capillaries in a large number of different types of purpura He found that the capillary loops in the nail bed were often distorted and irregular and sometimes branched and more important the capillaries did not contract normally on injury This work confirmed and extended the earlier observations of Leschke & Wittkower (1926) On the basis of an exhaustive study of the capillaries and the bleeding phenomena observed in a large number of different haemorrhagic disorders, Macfarlane formulated his hypothesis of haemostasis which explains why the bleeding time is prolonged when there is a capillary defect but not when blood coagulation is delayed According to this hypothesis small penetrating injuries, such as those inflicted in performing the bleeding time test damage mainly capillary blood vessels The initial result is free bleeding This is normally rapidly arrested by capillary con traction The extravasated blood in the wound then clots and, by the time the capillaries open again, a firm haemostatic clot has formed which prevents further bleeding In conditions in which blood coagulation is delayed bleeding is also arrested by capillary contraction although it may start again when the capillaries reopen if blood coagulation is sufficiently impaired to prevent the formation of a haemostatic clot In purpura, however the bleeding time is greatly prolonged because the capillaries do not contract normally on injury Plate 1 (A and B) shows the response of normal capillaries to injury and Plate 1 (C and D) the response of the capillaries in a case of non thrombocytopenic purpura (Macfarlane 1941)

Histological studies of the capillaries in purpura commonly show little significant change (Humble 1949 MacLeod 1933) Changes are how ever seen in anaphylactoid purpura and in purpura due to drug hyper sensitivity These changes will be considered later

ANAPHYLACTOID PURPURA

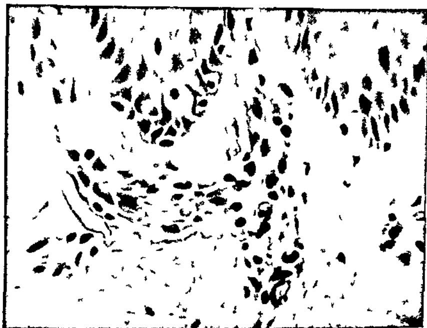
The first of the different types of purpura the pathogenesis of which I propose to discuss is anaphylactoid purpura—alternatively known as the Henoch–Schonlein syndrome Although true purpura (that is purpura with no surrounding macroscopically visible inflammatory reaction) is seen in this condition the characteristic rash is a slightly raised erythematous papule with a central haemorrhage

A very small number of cases are definitely due to food allergy This subject has been reviewed elsewhere (Ackroyd 1953) Perhaps the most striking of these cases is that described by Brown (1946) This patient developed the Henoch–Schonlein syndrome with purpura colic joint pains and haematuria whenever he ate tomato In the vast majority of cases however no cause is found

The widely held belief that the condition is commonly due to strepto coccal allergy receives little support from studies of the antistreptolysin titre in affected individuals Bywaters and his colleagues (1957) reported that although a history of a recent acute upper respiratory tract infection was common this was due to a β haemolytic streptococcus (as indicated by a raised antistreptolysin O titre) only in about a third of their cases This incidence was the same as that observed by them in a control group of patients It can be concluded therefore that unless the formation of anti streptolysin O antibody is abnormal in many patients with the Henoch–Schonlein syndrome (and there is no evidence that it is) then β haemolytic streptococcal infections are unlikely to be important in the aetiology of this condition or, if they are they can be so only in a relatively small proportion of cases

The platelet count in anaphylactoid purpura is normal or only slightly reduced and as no clotting defects have been reported the bleeding is presumably due to a capillary lesion Histological examination of the skin vessels in the area of the rash (see Plate 2) shows a marked perivascular inflammatory infiltration mainly with polymorphonuclear leucocytes and macrophages and some lymphocytes This change is seen around the small vessels of the corium Similar findings have been reported by Gairdner (1948) The vascular endothelium shows little change

Stefanini & Mednicoff (1954) claim that they have demonstrated a precipitin reaction between the sera of some patients with anaphylactoid purpura and extracts prepared from human aortic intima Cruickshank (1959) was however unable to confirm this finding Although this is the first time it has been claimed that an anti vascular endothelial factor can be demonstrated in patients with anaphylactoid purpura experimental



Anaphylactoid purpura section of an erythematous papule showing a dilated vessel in the corium with a perivascular infiltration of polymorphonuclear leucocytes and macrophages with some lymphocytes The endothelium appears normal (Reproduced from Ackroyd (1953) by permission of the *American Journal of Medicine*)

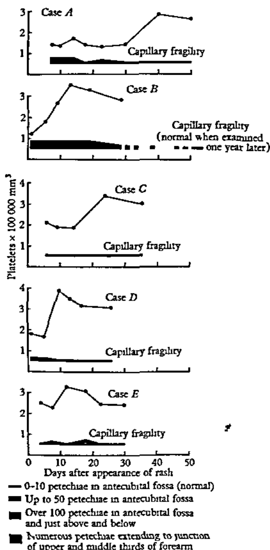


Fig 1 Changes in the platelet count and capillary fragility observed in five cases of mild uncomplicated rubella As indicated in the key the thickness of the lines representing capillary fragility is proportional to the number of haemorrhages produced in the Hess test performed by maintaining a pressure of 80 mm of mercury for 5 min (Redrawn from Ackroyd (1949*a*) and reproduced by permission of *The Quarterly Journal of Medicine*)

cytopenia and increased capillary fragility may persist for a long time after the termination of the acute illness there must be some factor which precipitates purpura at this stage Nothing is known of such a factor but the existence of a symptom free period before the onset of purpura suggests

work by many authors has shown that non thrombocytopenic purpura can be produced in animals by the administration of serum prepared against vascular endothelial cells This work has been reviewed and confirmed by Clark & Jacobs (1950) The significance of these observations is obscure and it is not certain whether they have any bearing on the pathogenesis of purpura in man However Stefanini & Mednicoff s (1954) work should it be confirmed, does suggest that the blood in patients with anaphylactoid purpura may contain an anti endothelial factor which is responsible for the vascular damage and resultant purpura Furthermore, it seems possible that the occurrence of an antigen–antibody reaction in the vessel walls might explain the inflammatory reaction seen around the vessels in purpuric areas in these patients I shall return to this subject later

PURPURA ASSOCIATED WITH INFECTIONS

True purpura occurring in association with infections is rare It has however, been described in association with a large number of both bacterial and viral infections Very little is known of the pathogenesis of this type of purpura Any hypothesis must take into account the facts (Ackroyd 1949*a*) that even in patients suffering from the same type of infection

1 Purpura may be associated with mild or severe infections In this way the condition differs from cases with a haemorrhagic exanthem which is seen only in severe infections

2 The purpura may be thrombocytopenic or non thrombocytopenic

3 Purpura may occur during the acute stage of the illness or during convalescence

At the height of almost any infection including even the common cold there is a fall in the platelet count (Bannerman 1924) This is often associated with a rise in capillary fragility Figure 1 shows these changes as they were seen in a small number of cases of very mild rubella The changes in the platelet count and in capillary fragility differed markedly both in degree and duration although, as far as could be determined clinically the infection in each patient was equally mild It seems probable therefore that these differences were due to differences in the susceptibility of the patient s tissues to the infection rather than to differences in the severity of the infection

It may well be that purpura occurs only in those patients with exceptionally high degrees of susceptibility resulting in such a rise in capillary fragility (and sometimes also a corresponding fall in the platelet count) that purpura develops Such an hypothesis would not however, explain the occurrence of purpura during convalescence for although thrombo

the antigenic specificity of the red cells of chickens infected with it (Gardner Wallace Dodd & Wright 1954)

In conclusion it must be stated that the cause of purpura associated with infections is unknown Those cases occurring in the acute stages may be due to an abnormal susceptibility of the patient s tissues to the infecting organism or its toxins Those occurring during convalescence may have an allergic basis similar to that which has been held responsible for the development of nephritis following streptococcal infections Whether thrombocytopenia can be due to modification of the patient s platelets by the infecting organism or its toxin so that the platelets become antigenic and stimulate the formation of antibodies which can cause their lysis is not known There are several objections to such an hypothesis

IDIOPATHIC THROMBOCYTOPENIC PURPURA

The cause of the thrombocytopenia

In recent years the idea has gained ground that the low platelet count in idiopathic thrombocytopenic purpura is due to the action of a circulating anti platelet factor in the patient s plasma This hypothesis is however by no means new and it may well be that some such factor was in Hayem s mind when he suggested (Hayem 1896) that thrombocytopenia was due to agglutination of platelets into masses which produced haemorrhagic infarcts

Dans le *purpura hemorragica* la diminution dans le nombre des hematoblastes ne me parait pas etre la consequence d un arret dans la formation de ces élements Mes observations m ont conduit a admettre dans cette affection la penetration dans le sang d une substance alterant les hematoblastes et les precipitant. Ce sont les hématoblastes alteres qui reunis en amas seraient la cause des hémorragies et mieux des *infarctus* hemorragiques

Research in this field received a tremendous impetus from the courageous experiment of Dr William Harrington who transfused himself with blood from a patient with idiopathic thrombocytopenic purpura and within a few hours developed the disease He recovered after a brief but alarming illness It was subsequently shown that if normal plasma is injected into normal recipients there is a variable transient fall in the platelet count (as much as 50% of the original count in some cases (Stefanini & Chatterjea 1952)) If the plasma is taken from some patients with idiopathic thrombo cytopenic purpura the fall may be greater and may persist for longer (Harrington Minnich Hollingsworth & Moore 1951 Stefanini & Dameshek 1955) Occasionally as in Harrington s original experiment the fall in the platelet count is accompanied by purpura (Harrington *et al*

an analogy with nephritis following streptococcal infections In this connexion it is interesting to note that the period of maximal incidence of purpura following scarlet fever coincides almost exactly with that of post scarlatinal nephritis (Box, 1933) It seems possible therefore that purpura occurring during convalescence from acute infections may be due to a mechanism akin to that which causes nephritis following streptococcal infections

Before concluding this discussion it is necessary to consider some experimental observations made by Kirstner & Stefanini (1956) These workers incubated rabbit platelets with various bacterial filtrates and with suspensions of Newcastle disease virus They then attempted to immunize these animals with their own incubated platelets They found that after immunization some of the animals developed thrombocytopenia when injected with the appropriate bacterial filtrate alone Attempts to produce similar results with Newcastle disease virus were unsuccessful

It is not clear whether these findings have any counterpart in human pathology They might be taken to indicate that purpura associated with infections is due to an action of the infecting organism or its toxin on the patient s platelets modifying them and so rendering them antigenic This hypothesis is difficult to accept for it does not readily explain several of the observed facts about this type of purpura For instance it implies that before purpura develops the patient must have been infected with the same or a very closely related organism on at least one or probably several previous occasions, for in the absence of previous infection the formation of antibodies against the modified platelets could not occur Although therefore this hypothesis might explain the occurrence of purpura in the acute stages of diseases in which repeated infections are common as for example streptococcal infections it will not explain purpura associated with the acute specific fevers which rank high among the causes of this type of purpura but in which second infections are rare Furthermore if purpura is an occasional result of repeated infections with organisms to which the body does not develop lasting immunity then it would be expected by analogy with purpura due to drug hypersensitivity (see p 232) that after the first attack of purpura, every subsequent infection with the same organism would be accompanied by purpura If this does occur it must be extremely rare

Another objection to this hypothesis is that although virus diseases are common amongst the causes of this type of purpura Kirstner & Stefanini (1956) did not succeed in producing thrombocytopenia when they used platelets which they had attempted to modify with a virus suspension although they used Newcastle disease virus which has been shown to alter

type may occur even if the child is born at a time when the mother has no purpura because her lesion has been controlled by splenectomy (Epstein Lozner Cobbey & Davidson 1950 Robson & Walker 1951 Harrington *et al* 1953) The condition thus resembles thrombocytopenia induced by transfusion of blood from patients with idiopathic thrombo cytopenic purpura and suggests therefore that the thrombocytopenia in the infant may be due to transplacental passage of the anti platelet factor in the mother s blood

The second observation is based on estimations of the time of survival of platelets transfused into patients with different types of thrombocyto penic purpura It has been found by several groups of workers that platelet survival in idiopathic thrombocytopenic purpura is very much shorter than it is in cases of secondary amegakaryocytic thrombocytopenic purpura in which platelet survival is believed to be normal (Stefanini Chatterjea Dameshek Zannos & Santiago 1952 Hirsch & Gardner 1952 Sprague *et al* 1952 Stefanini & Dameshek 1955) The shortened platelet survival time in a patient with idiopathic thrombocytopenic purpura is clearly shown in Fig 3

It has been observed that when patients with secondary amegakaryo cytic thrombocytopenia are repeatedly transfused platelet survival becomes progressively shorter (Hirsch & Gardner 1952 Stefanini Dameshek & Adelson 1952) A typical example of this is shown in Fig 4 It is difficult to see how this finding can be explained except on the assumption that the transfused platelets have acted as antigens and that the progressive shortening in the survival time of the transfused platelets is due to a progressive rise in the titre of an anti platelet antibody If it is accepted that platelets can act as antigens then the possibility has to be considered that the anti platelet factor in idiopathic thrombocytopenic purpura may be an auto antibody

Experiments and observations such as those I have just quoted led to an intensive world wide search for anti platelet antibodies in the blood of patients with idiopathic thrombocytopenic purpura and a large number of papers were written describing methods for demonstrating platelet agglutinins in this condition The significance of the findings of the different workers in this field proved difficult to interpret This was very clearly demonstrated by an investigation the results of which I have been able to see through the courtesy of Dr Stefanini (personal communication) A group of sera the origin of which was known only to the originator of the experiment was distributed to a number of haematologists who had devised different methods for demonstrating platelet agglutinins The sera consisted of samples from ten patients with idiopathic thrombocytopenic

1951) Typical changes in the platelet count resulting from transfusion of blood or plasma from patients with idiopathic thrombocytopenic purpura into normal recipients are shown in Fig 2 The thrombocytopenic factor apparently persists after a remission has been induced by splenectomy although its disappearance after spontaneous or cortisone induced remissions has been observed (Sprague, Harrington, Lange & Shapleigh, 1952)

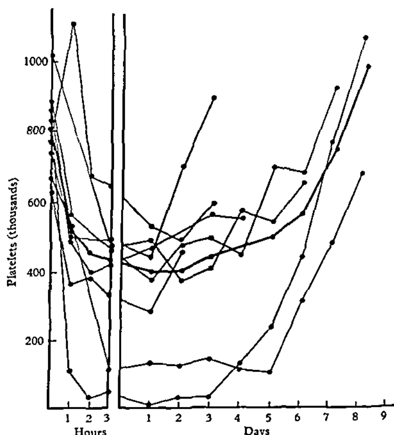


Fig 2 Effects on the platelet counts of normal individuals of transfusing 500 ml of citrated blood or its plasma equivalent from eight patients with idiopathic thrombocytopenic purpura The mean effect is indicated by the heavy line (Reproduced from Harrington *et al* (1951) by permission of *The Journal of Laboratory and Clinical Medicine*)

These observations seem to provide clear evidence of the existence of an anti platelet factor in the blood of some patients with idiopathic thrombocytopenic purpura There are at least two other observations which also provide good evidence for the existence of such a factor

The first is that some infants of mothers with idiopathic thrombocytopenic purpura are born with thrombocytopenic purpura from which the child fairly soon recovers Congenital thrombocytopenic purpura of this

to refer to this non immune platelet agglutination as clumping It would seem probable that this spontaneous clumping was in part responsible for the contradictory results obtained with the different tests for platelet agglutinins

Subsequent work has centred largely on three methods for demonstrating platelet antibodies One is Dausset s method of demonstrating immune platelet agglutinins active at 37 C In this immense care is taken to minimize errors due to spontaneous clumping (Dausset & Colin 1958 Dausset 1958*a*) The two remaining methods are not influenced by

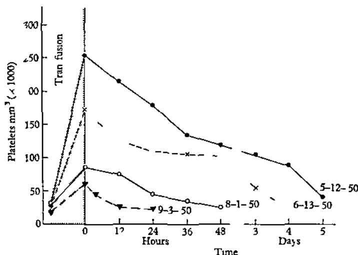


Fig 4 Effect of repeated platelet transfusions into a pat ent with amegakaryocytic thrombocytopenia due to hypoplastic anaemia The e is a progressive r duction in the n t l rise in the platelet count caused by each transfus on and also in the time of survival of the transfused platelets (Reproduced from Stefanini Dameshek & Adel on (1952) by permission of *The P oceedings of the Soci ty of Experimental Biology and Medicine*)

spontaneous platelet clumping if this occurs They are the antiglobulin consumption test (Moulinier 1955) and the specific mixed antiglobulin technique (Coombs Marks & Bedford 1956 Chalmers Coombs Gurner & Dausset 1959)

In the antiglobulin consumption test the thrombocytopenic patient s own platelets if any can be obtained are washed and then incubated with anti human globulin serum Alternatively normal platelets which have previously been treated with papain are incubated with serum from a patient with idiopathic thrombocytopenic purpura in an attempt to sensitize them The platelets are then washed and incubated with antiglobulin serum With platelets prepared in either way a fall in antiglobulin titre is taken to indicate the presence of immune globulin on the surface of the platelets

purpura and from five normal subjects who had never been transfused No two methods gave identical results nor was any of them capable of distinguishing the normal from the pathological sera Moreover every

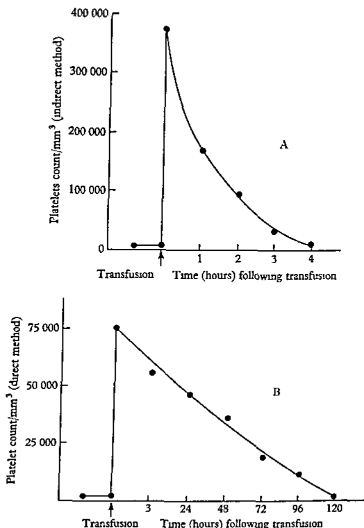


Fig 3 A Survival of platelets from normal whole blood transfused into a patient with idiopathic thrombocytopenic purpura B Survival of platelets from normal whole blood transfused into a patient with secondary amegakaryocytic thrombocytopenia *due to* aplastic anaemia (Reproduced from Sprague *et al* (195) by permission of *The Journal of the American Medical Association*)

method demonstrated platelet agglutinins in at least one of the five normal sera One method demonstrated them in three The difficulty with using platelet agglutination as a method of demonstrating antibodies to platelets is that agglutination is a normal function of platelets I propose hereafter

nated red cells and in this way the fact that they have adsorbed antibody will be demonstrated Typical positive and negative findings by this method are shown in Plate 3

Recent publications by Coombs and by Dausset have made it possible to draw some tentative conclusions as to the relative values of these three techniques

(1) The sera of patients who have received many transfusions and in whom the survival times of transfused platelets may be expected to be reduced appear to contain antibodies which can be demonstrated by Dausset s agglutination technique (Dausset 1957 1958*a*) Two sera giving positive results with this technique were also tested with Coombs mixed antiglobulin technique Each gave a positive reaction (Chalmers *et al* 1959) It would seem therefore that either method can be used to demonstrate anti platelet antibodies in such patients

(2) No platelet antibodies have been demonstrated in the sera of patients with idiopathic thrombocytopenic purpura by the mixed antiglobulin technique (Coombs Marks & Bedford 1956 Chalmers *et al* 1959) In a paper read at the 1957 Haematological Congress at Copenhagen Dausset (1957) claimed that his agglutination technique gave positive results in some cases of idiopathic thrombocytopenic purpura but he did not make this claim in a paper on antibodies to platelets which he read in 1958 at the Congress of Allergy in Paris (Dausset 1958*a*) It may therefore probably be concluded that antibodies to platelets in patients with idiopathic thrombocytopenic purpura cannot be demonstrated with certainty by either method

(3) Dausset (1957 1958*a*) has claimed that anti platelet antibodies in the sera of some patients with idiopathic thrombocytopenic purpura can be demonstrated with the antiglobulin consumption test Whether this test will prove to give sufficiently reproducible results to make it of value in the elucidation of the immunological mechanisms underlying idiopathic thrombocytopenic purpura remains to be seen The technique appears to be difficult and Dr Coombs tells me (personal communication) that he has so far been unable to confirm Dausset s claim

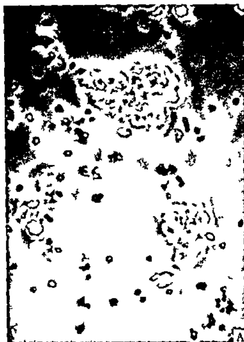
In conclusion it can be said that although serological tests have failed to shed much light on the pathogenesis of idiopathic thrombocytopenic purpura collateral evidence strongly suggests that the low platelet count in this condition is due to the action of an anti platelet factor in the patient s plasma

The principle of the specific mixed antiglobulin technique is indicated in Fig 5 A suspension in saline is prepared of the thrombocytopenic patient's own washed platelets if these can be obtained Alternatively normal trypsinized platelets are used (Chalmers *et al* 1959) and are incubated with the patient s serum They are then washed and suspended in saline A suspension in saline is also prepared of human red cells which have been sensitized with human incomplete antibody and sub

	Platelets Unsensitized	Red cells sensitized with human inc antibody	Platelets sensitized with human antibody	Red cells sensitized with human inc antibody
Cell suspensions				
Rabbit anti human globulin ( )—( )	Only the red cells possess adsorbed globulin Addition of antiglobulin gives		Both cell types possess a common antigen (adsorbed globulin) Addition of antiglobulin gives	
Reaction				
	Specific red cell agglutination No mixed agglutination		Mixed specific agglutination	

Fig 5 Schematic representation of specific mixed agglutination of sensitized platelets and sensitized erythrocytes by antiglobulin serum in the specific mixed antiglobulin test (inc = incomplete) (Reproduced from Coombs Marks & Bedford (1956) by permission of *The British Journal of Haematology*)

sequently washed The platelet and red cell suspensions are mixed and anti human globulin serum is added The erythrocytes will, of course agglutinate The platelets if they have adsorbed antibody on their surface (i e if they are sensitized) will also agglutinate but in addition the agglutinated platelets will become firmly agglutinated with the agglutinated red cells If spontaneous clumping of the platelets has occurred this will not affect the result for if the platelets have not adsorbed antibody the clumps of platelets will not become agglutinated with the agglutinated red cells If the platelets have adsorbed antibody and have undergone spontaneous clumping the clumped platelets will become agglutinated with the aggluti





Spec fic m d ant globul n r a t on A \ gat e e ult th rythrocyt s are agglutinated and some of the platelets re cl mped togethe but no m ed agglutination of platelets and erythrocytes has occurred (the plat let appea d rk a d th rythrocytes b ght) B I os ti e result the pl telets and erythro tes how m xed agglut n t o that is they r agglut nated togeth r (the pl telets ppear da k nd the rythrocyte bright) (R produ ed from Chalmers *et al* (1959) by perm s on of *Tl e British J rnal of H ematol gy*)

(*F ing p* 232)

PURPURA DUE TO DRUG HYPERSENSITIVITY

Non thrombocytopenic purpura

Purpura due to drug hypersensitivity may be thrombocytopenic or may be associated with a normal platelet count In my experience, the drug which is by far the most commonly implicated is the open chain ureide carbromal (synonym adalin) This is diethyl bromo acetyl carbamide Its structural formula is shown in Fig 6 In sensitized patients it causes a non thrombocytopenic type of purpura It is of interest to note that the commonest cause of thrombocytopenic purpura due to drug hypersensitivity is another open chain ureide sedormid (see p 233 and Fig 7) This suggests that there must be some feature of the general structure of the two molecules which confers upon them this peculiar ability to cause hypersensitivity reactions involving the vascular endothelium It is also of interest that the differences in the side chains in the two molecules should confer upon the sedormid molecule an ability to cause a hypersensitivity reaction which involves also the platelets

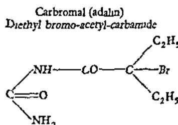


Fig 6 The chemical formula of carbromal

Purpura due to carbromal is associated with a dermatitis which often itches The purpura is consequently seen against an erythematous back ground with some desquamation of epidermal cells Serious bleeding rarely if ever occurs The condition invariably recovers in a week or two if the drug is withdrawn but rapidly recurs if the patient should take the drug again

The diagnosis can be confirmed by patch testing typical results of which are shown in Plate 4

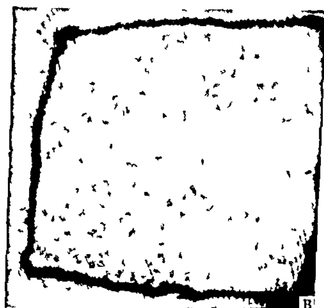
No coagulation defects have been described and as the platelet count is normal the purpura would appear to be due solely to a capillary lesion The vessels in the affected areas have been studied histologically (see Plate 5) The small vessels just beneath the epidermis show a marked perivascular infiltration with lymphocytes polymorphonuclear leucocytes and macrophages The endothelial cells show no striking changes A similar perivascular inflammatory reaction has been reported in a case of non thrombocytopenic purpura due to menthol (Highstein & Zeligman 1951) As might be expected from the fact that in carbromal sensitivity the purpura is associated with dermatitis in this condition there are also inflammatory cells in the dermis just below the rete malpighii and in places extending





Photomicrographs of skin from an are of purpu a in a pati nt uffe ng from carb omal purpura A Low power ew howing pe scular nflammatory reaction There i also small number of infl mmatory lls n the dermis wh ch extend n pl ces nto the rete m lpigh i B High power vi w showing det ls f p rivascular inflamm tory eaction The cells are ma nly polymorphonucl ar leucocytes lymphocyt s nd macrophages The end thelial cell appear norm l





Results of patch testing a carbromal sensitive patient A Control using solvent (propyl ne glycol) alone The skin appears normal B Result of testing with a suspension of carbromal crystals in a saturated solution in propylene glycol The area of skin which has been in contact with carbromal shows numerous petechial haemorrhages The skin in this area is slightly erythematous and there is some epithelial desquamation

into it I shall consider the significance of the perivascular inflammatory reaction when describing a similar reaction seen in thrombocytopenic purpura due to sedormid

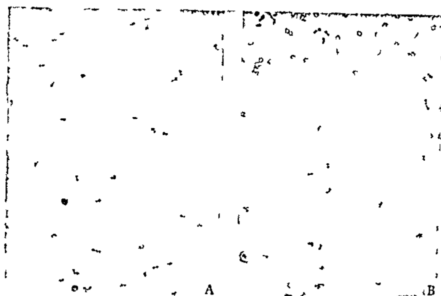
Thrombocytopenic purpura

Thrombocytopenic purpura due to drug sensitivity has occurred as a rare complication of treatment with a very large number of different drugs This subject has been reviewed elsewhere (Ackroyd 1955*a* 1958) The drugs which have most commonly caused this type of purpura are sedormid quinidine quinine sulphonamides arsenobenzol compounds and gold salts The purpura occurs only when the appropriate drug is taken by a sensitized individual Unless the drug is one which is liberated slowly from a depot (e g gold salts) or unless fatal haemorrhage occurs into a vital organ the purpura rapidly recovers when the drug is withdrawn only to recur should the drug be taken again Otherwise the clinical picture is indistinguishable from that of idiopathic thrombocytopenic purpura

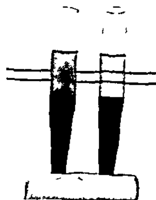
Two immunological mechanisms have been observed in cases of thrombocytopenic purpura due to drug hypersensitivity In the first the drug causes *in vitro* only platelet agglutination and lysis In the second the drug causes in addition the formation of an immune precipitate The first originally analysed in three cases due to sedormid and since demon strated in cases due to other drugs would appear to be much the more common (Ackroyd 1949*b* *c* *d* 1951 1954 1958) The second has been analysed only in one case due to antazoline (Ackroyd 1955*b* 1958 1960*a* *b*) In describing the two different mechanisms I shall refer only to the cases in which they were originally analysed namely those due to sedormid and antazoline The chemical formulae of these two drugs are shown in Fig 7

Thrombocytopenic purpura due to hypersensitivity to sedormid An anti platelet substance dependent for its action on the presence of sedormid can be demonstrated in the blood of patients who have recovered from sedormid purpura if the drug is added to the patient s blood or platelet rich plasma in the presence of anticoagulants The quantity of anti coagulant should be the minimum required to prevent coagulation Plate 6 (A and B) shows the effect of adding sedormid to the heparinized platelet rich plasma of a sensitized patient Plate 6 A shows the control preparation to which saline only has been added Agglutination of the platelets and later lysis (Plate 6 B) followed the addition of sedormid in saline to a sample of platelet rich plasma taken from the same specimen from which the control was taken The demonstration of platelet agglutina tion and lysis *in this way is technically difficult* (Ackroyd 1949*d* 1951)

PLATE 6



Platelet agglutination and lysis in heparinized platelet rich plasma from a sedormid sensitive patient A Plasma + saline No platelet agglutination or lysis B Plasma + sedormid in saline There is considerable platelet lysis The incompletely lysed remnants of some of the platelets can still be seen Most of the remaining platelets are agglutinated (Reproduced from Ackroyd (1952) by permission of *Progress in Allergy*)



Clearing of citrated plasma by sedormid in blood from a sedormid sensitive patient The tubes have been allowed to stand to permit sedimentation of the red and white cells C Blood + isotonic sodium citrate solution The supernatant plasma is opalescent mainly owing to the presence of large numbers of platelets D Blood + solution of sedormid in isotonic sodium citrate The plasma is clear and transparent as a result of agglutination and lysis of platelets (Reproduced from Ackroyd (1949*d*) by permission of *Clinical Science*)

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Thrombocytopenic purpura due to drug sensitivity has occurred as a rare complication of treatment with a very large number of different drugs This subject has been reviewed elsewhere (Ackroyd 1955*a* 1958) The drugs which have most commonly caused this type of purpura are sedormid quinidine quinine sulphonamides arsenobenzol compounds and gold salts The purpura occurs only when the appropriate drug is taken by a sensitized individual Unless the drug is one which is liberated slowly from a depot (e g gold salts) or unless fatal haemorrhage occurs into a vital organ the purpura rapidly recovers when the drug is withdrawn only to recur should the drug be taken again Otherwise the clinical picture is indistinguishable from that of idiopathic thrombocytopenic purpura

Two immunological mechanisms have been observed in cases of thrombocytopenic purpura due to drug hypersensitivity In the first the drug causes *in vitro* only platelet agglutination and lysis In the second the drug causes in addition the formation of an immune precipitate The first originally analysed in three cases due to sedormid and since demonstrated in cases due to other drugs would appear to be much the more common (Ackroyd 1949*b c d* 1951 1954 1958) The second has been analysed only in one case due to antazoline (Ackroyd 1955*b* 1958 1960*a b*) In describing the two different mechanisms I shall refer only to the cases in which they were originally analysed namely those due to sedormid and antazoline The chemical formulae of these two drugs are shown in Fig 7

Thrombocytopenic purpura due to hypersensitivity to sedormid An anti platelet substance dependent for its action on the presence of sedormid can be demonstrated in the blood of patients who have recovered from sedormid purpura if the drug is added to the patient s blood or platelet rich plasma in the presence of anticoagulants The quantity of anti coagulant should be the minimum required to prevent coagulation Plate 6 (A and B) shows the effect of adding sedormid to the heparinized platelet rich plasma of a sensitized patient Plate 6 A shows the control preparation to which saline only has been added Agglutination of the platelets and later lysis (Plate 6 B) followed the addition of sedormid in saline to a sample of platelet rich plasma taken from the same specimen from which the control was taken The demonstration of platelet agglutination and lysis in this way is technically difficult (Ackroyd 1949*d* 1951)

A much simpler method is to add the patient s whole blood to a solution of the drug in an anticoagulant A control tube in which the blood is diluted only with anticoagulant is also put up After mixing the tubes are allowed to stand and the red and white cells to sediment If the patient is sensitive to sedormid the number of platelets in the supernatant plasma in the sedormid preparation will be lower than in the control The fall in the platelet count is due partly to sedimentation of agglutinated platelets and partly to platelet lysis If the patient is very sensitive to the drug the supernatant plasma in the sedormid preparation will contain practically no platelets and will be clear and transparent whereas it will be

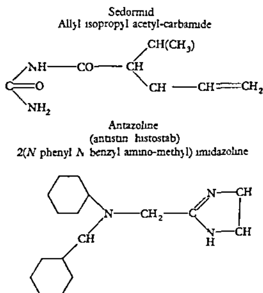


Fig 7 Chemical formulae of sedormid and antazoline

opalescent in the control preparation owing to the presence of large numbers of platelets (Ackroyd 1949*d*) An example of such a finding in a highly sensitized patient is shown in Plate 6 (C and D)

Complement is fixed during platelet lysis by sedormid but complement is not fixed if sedormid is added to the patient s serum alone Platelets also are necessary (Ackroyd 1951)

If normal platelets are suspended in the serum of a sensitized patient and sedormid is added complement is fixed but if the patient s platelets are suspended in normal serum the addition of sedormid does not cause complement fixation It is clear therefore that the abnormality lies in the serum of sensitized patients and not in their platelets (Ackroyd 1951) This serum factor is a γ globulin (Ackroyd 1958) Since it is a γ globulin

and since it causes platelet lysis with fixation of complement it would seem almost certain that it is an antibody

The union of sedormid with platelets and this antibody is remarkably labile If platelets and sedormid are incubated with inactivated serum from a sensitized patient (i e. serum which has been heated to destroy its complement) and the platelets are then washed in saline the platelets will be found to have been washed free of both sedormid and the antibody If however the platelets in such an experiment are washed, not in saline but in a saturated solution of sedormid in saline the antibody will remain in contact with the platelets (Ackroyd 1954) But if such platelets are dialysed against saline and the platelet suspension is centrifuged, all the antibody will be found in the supernatant fluid, the sedormid having dialysed away from union with the platelets and antibody and the antibody having separated from the platelets (Ackroyd 1954) When therefore one comes to consider the role of sedormid in this reaction it is clear that it acts as a link between platelets and antibody The most probable explanation for such a finding is that sedormid acts as a hapten conferring antigenic properties on the platelets the sedormid molecule supplying the determinant group by which the antibody is attached to the platelet. This concept is shown schematically in Fig 8 It explains why after dialysis as in the experiment described above the antibody is found free in the supernatant fluid for when the hapten (sedormid) is removed by dialysis the platelets, no longer being antigenic, must separate from the antibody This concept also explains why thrombocytopenia develops only when the drug is taken by a sensitized individual for it is only then that the hapten is available to render the platelets antigenic and so to enable the antibody to react with them and cause their lysis with resultant thrombocytopenia

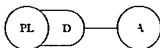


Fig 8 Diagram showing how the antibody (A) in the sera of sedormid sensitive patients may combine with platelets (PL) if these are rendered antigenic by union with the drug sedormid (D) which probably acts as a hapten.

Labile compounds if they have antigenic properties, are likely to be only weakly antigenic—probably because they do not often remain in contact with the antibody forming tissues long enough to stimulate antibody formation (see Haurowitz Tunca & Schwerin, 1943) It has been shown that sedormid combines with the platelets of normal individuals when these are in contact with serum (Ackroyd 1958) or with traces of plasma (Dausset, 1958*b*) from the same individual As the combination of sedormid with platelets, even in the presence of antibody is extremely labile it would seem probable that it is only a very weak antigen It is suggested that the very lability of the platelet sedormid antigen may explain why so few of

those taking the drug develop purpura for only those whose antibody forming mechanisms are stimulated by this very labile antigen will manufacture the antibody and so develop purpura

Thrombocytopenic purpura due to hypersensitivity to antazoline The following description is based on an investigation of a single case of thrombocytopenic purpura due to the antihistaminic drug antazoline in which the addition of the drug to the patient s blood after recovery, caused not only platelet agglutination and lysis but also immune precipitate formation Precipitate formation by drugs must be an extremely rare phenomenon Hoigné (1958) has claimed that a very slight increase in opalescence detectable only with specially sensitive optical apparatus is commonly produced when a drug is added to the serum of a patient who is sensitive to it but apart from this precipitate formation by a drug appears to have been observed only twice before and in neither case was the mechanism analysed (see below)

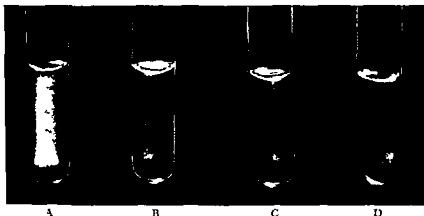


Fig 9 Diagram showing how the antibody (A) in the serum of an antazoline sensitive patient may combine both with platelets (PL) and protein (PR) if these are rendered antigenic by union with the drug antazoline (D) which probably acts as a hapten

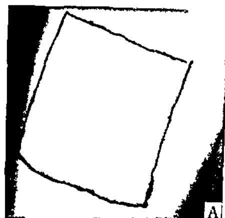
The immunological mechanism by which antazoline causes platelet lysis appears to be identical with that described for sedormid (Ackroyd 1960*a*)

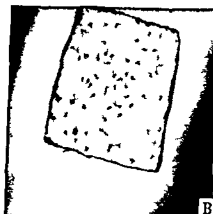
The precipitate caused by the addition of antazoline to the patient s serum is shown in Plate 7 Complement is fixed during precipitate formation (Ackroyd, 1955*b* 1960*a*)

It has been shown by methods described elsewhere (Ackroyd 1958 1960*b*) that the antibody which causes precipitate formation is almost certainly identical with the antibody which causes platelet lysis Precipitate formation probably results from the interaction of this antibody with plasma protein rendered antigenic by union with the drug which acts as a hapten Platelet lysis probably results from the interaction of this same antibody with platelets rendered antigenic by union with the drug The mechanism is shown schematically in Fig 9 The concept of one antibody reacting with two different antigens is contrary to current immunological theory It seems however altogether probable in this case since both antigens—protein and platelet—have the same hapten determinant group and may well therefore behave immunologically as if they were identical



Immun precipitate form tion in th serum of an antazoline sens ti e patient A Patient s serum + antazoline in sal ne A floccular precip tat has form d B Patient s serum + s line No precip tate C Control serum + antazol ne in sal ne No prec p tate D Control serum + saline No prec p tate (Reprodu d from Ackroyd (1955*b*) by permission of *Le S ng*)





Results of patch testing a sedormid sensitive patient A Control using solvent (propylene glycol) alone The skin appears normal B Result of testing with a suspension of sedormid crystals in a saturated solution in propylene glycol The area of skin which has been in contact with sedormid is closely packed with petechial haemorrhages (Reproduced from Ackroyd (1949*b*) by permission of *Clinical Science*)





Photomicrographs of skin from an area of purpura produced by patch testing a sedormid sensitive patient C Low power view showing perivascular inflammatory reaction D High power view showing details of perivascular inflammatory reaction The cells are mainly polymorphonuclear leucocyte lymphocytes and macrophages The endothelial cells appear normal (Reproduced from Ackroyd (1958) by permission of Blackwell Scientific Publications Oxford)

The relationship of the two mechanisms to the different drugs which have caused thrombocytopenic purpura

In the above description sedormid has been given as an example of a drug causing platelet lysis alone and antazoline as one causing in addition immune precipitate formation The former appears to be much the commoner mechanism for apart from the case due to antazoline referred to above of all the numerous cases of thrombocytopenic purpura due to drug hypersensitivity which have been investigated by different workers only two other cases associated with immune precipitate formation have been described In one of these the causative drug was sedormid (Miescher & Miescher 1952) and in the other quinidine (López Garcia & Sainz de la Maza 1951) Since many cases of thrombocytopenic purpura due to these two drugs have been described in which precipitate formation was not observed it would appear that it is the idiosyncrasy of the patient rather than the chemical nature of the drug which determines whether or not precipitate formation will occur

The capillary lesion in thrombocytopenic purpura due to drug hypersensitivity

This has been studied by patch testing and also by histological examination of purpuric areas of skin The results of patch testing a sedormid sensitive patient are shown in Plate 8 (A and B) The drug has caused the appearance of numerous petechial haemorrhages in the area of skin to which it has been applied These were produced without any change in the patient s platelet count This observation which has been made in two patients who were sensitive to sedormid, strongly suggests that the capillary lesion is independent of platelet lysis

The histological appearances of the purpuric rash caused by patch testing a sedormid sensitive patient are shown in Plate 8 (C and D) The striking feature of these photomicrographs is the perivascular inflammatory reaction around the small vessels of the corium The inflammatory cells are in the main polymorphonuclear leucocytes lymphocytes and macrophages No significant changes are seen in the vascular endothelial cells The picture therefore closely resembles that seen in anaphylactoid purpura and in purpura due to hypersensitivity to carbromal and to menthol

The significance of the perivascular inflammatory reaction seen in anaphylactoid purpura and in thrombocytopenic and non thrombocytopenic purpura due to drug hypersensitivity

The significance of this perivascular inflammatory reaction is obscure for such appearances are not uncommonly seen in a wide variety of skin diseases However, in view of the fact that in all the conditions considered here the perivascular cuffing has been found in association with a known vascular lesion it may well be as I suggested in connexion with anaphylactoid purpura, that it represents a response to an antigen-antibody reaction occurring in the vessel wall

In considering thrombocytopenic purpura due to drug hypersensitivity it is tempting to suggest since vascular endothelial cells are immunologically so closely related to platelets, that they also may form an antigenic combination with the drug and react with the antibody which causes platelet lysis In non thrombocytopenic purpura due to drugs it may be suggested that the vascular lesion is due to interaction of an antibody with endothelial cells rendered antigenic by union with the causative drug These suggestions are highly speculative I have performed some experiments with serum from two cases of sedormid purpura which suggest that such a reaction between the sedormid antibody sedormid and vascular endothelial cells may in fact occur, but the results so far are not sufficiently clear cut to permit any firm conclusion to be drawn from them If, however, such reactions between antibody and endothelial cells do occur then it may be that the perivascular cuffing seen in these conditions and also in anaphylactoid purpura is a response to an antigen–antibody reaction occurring in the vessel wall

ABNORMAL BLEEDING ASSOCIATED WITH DYSPROTEINAEMIA

There are five conditions in which pathological bleeding occurs in association with the presence of abnormal proteins in the blood

1 Myelomatosis
2 *Amyloidosis*
3 Hyperglobulinaemic purpura
4 Macroglobulinaemia
5 Cryoglobulinaemia

Myelomatosis

This condition has been too well described elsewhere to merit any further description here It is well known as a disease which is associated with the

presence of abnormal proteins in the blood It is perhaps not so well recognized that purpura is not uncommon in myelomatosis especially in the later stages of the disease

Amyloidosis

Amyloidosis is frequently although not invariably associated with a raised level of globulins in the plasma (Bywaters & Glynn 1957) This is true even in the primary form of the disease In the familial type of primary amyloidosis an abnormal globulin migrating between the β and α_2 areas can be demonstrated on free electrophoresis It appears to be a lipoprotein (Rukavina *et al* 1956) Primary amyloidosis is included in this section because in addition to the protein abnormality it is not uncommonly associated with non thrombocytopenic purpura (Propp Scharfman Beebe & Wright, 1954) Purpura which may be thrombocytopenic or non thrombocytopenic is also common in cases of secondary amyloidosis but here the relationship of the amyloid to the purpura is sometimes difficult to establish as the causative condition e g myelomatosis lymphadenoma etc may be associated with purpura even in the absence of amyloidosis

Hyperglobulinaemic purpura

This is a very rare benign condition characterized by relapsing purpura which is seen chiefly on the legs As the purpura clears it tends to leave pigmented spots on the skin so that ultimately after many attacks of purpura the affected areas become deeply pigmented Serious bleeding does not occur and the condition may persist for many years It does not apparently shorten life (Waldenstrom 1952*a* *b*) There are no abnormal cells in the bone marrow Apart from the frequent occurrence of slight enlargement of the lymph glands and a moderate normochromic anaemia the only significant abnormality is a great increase in apparently normal γ globulin in the serum (Waldenstrom 1952*a* *b* Stefanini & Dameshek 1955)

Macroglobulinaemia

Macroglobulins are large globulins having a sedimentation constant of 15 or more Svedberg units They are found in small quantities (up to 3%) in normal sera (MacKay Erikson Motulsky & Volwiler 1956) In fact the protein properdin is a macroglobulin (Pillemer *et al* 1954)

Waldenstrom (1944 1952*a* *b* 1958) has described what appears to be a specific reticulosis in which there is a large increase in macroglobulins in the patient s serum In this condition the lymph glands liver and spleen may be enlarged The blood shows a progressive normochromic anaemia In some cases there is also a moderate thrombocytopenia and an increase

in lymphocytes in the peripheral blood These cells are characteristically found in greatly increased numbers in the bone marrow in which there may also be a moderate increase in plasma cells

In other cases the increase in macroglobulins in the blood is secondary to some other reticulosis (Waldenstrom 1958 Martin & Close, 1957) Lymphatic leukaemia and lymphosarcoma are perhaps the reticuloses most commonly associated with macroglobulinaemia

Because of the high concentration of the abnormal globulin the erythrocyte sedimentation rate is high and the viscosity of the serum is greatly increased

Although the presence of large amounts of macroglobulins in the blood does not necessarily cause symptoms (Martin & Close 1957) macroglobulinaemia may be associated with a haemorrhagic syndrome The *bleeding occurs mainly from mucosal surfaces, particularly in the nose and* mouth Skin purpura does occur but is rare

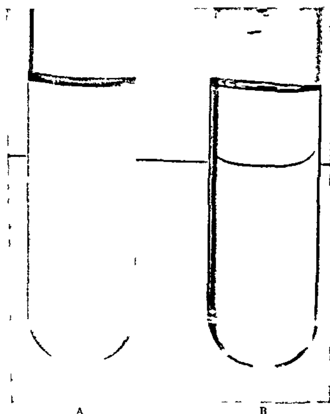
Macroglobulinaemia can be diagnosed only by means of ultra centrifugation *However although not completely reliable the euglobulin test (also* referred to as the Sia (Sia & Wu 1921) or Brahmachari (Brahmachari & Sen 1923) test) is thought to be a valuable screening test (Waldenstrom 1944 1952*b* 1958) One volume of serum is added to at least twenty volumes of distilled water Normal serum produces at most a faint haze Macroglobulinaemic sera produce a dense precipitate which fairly rapidly falls to the bottom of the tube The precipitated protein readily redissolves if a solution of sodium chloride is added Plate 9 shows a positive euglobulin test Any serum in which the concentration of globulin is sufficiently high will give a dense white precipitate in this test soluble in added salt solution For this reason the test is often positive in kala azar in which there is an increase in normal γ globulin It may be possible to distinguish between normal and macroglobulins in this test for according to Waldenstrom (1958) sedimentation of the precipitated protein does not occur in kala azar whereas it is characteristic of macroglobulinaemia that the precipitated protein rapidly falls to the bottom of the tube

Cryoglobulinaemia

Cryoglobulins are globulins which come out of solution when plasma or serum is cooled below 37° C and redissolve on warming

Most cryoglobulins have the sedimentation constant of normal globulins that is they have the same molecular weight Some cryoglobulins are macroglobulins

Cryoglobulins do not occur in normal plasma (Lerner Barnum & Watson 1947)



The S a (euglobulin) test in macroglobulinaem a A Tube showing opal scence due to pr cip tation of macroglobulin f om 1 o ml of s um added to o ml of d st lled wate Th phot gr ph was taken before sediment ti n of the prec p tated protein had occur d B Tube howing re solution of precipitated mac oglobulin as a result of the add t on of a solution of odium chlor de

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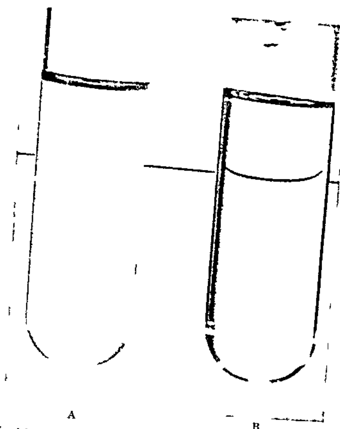
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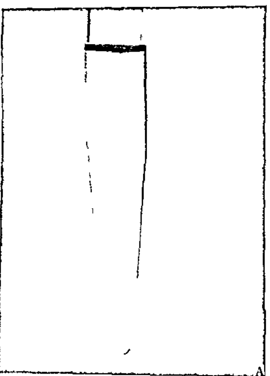
The Sia (euglobulin) test in macroglobulinaemia. A. Tube showing opalescence due to precipitation of macroglobulin from 1·0 ml of serum added to 20 ml of distilled water. The photograph was taken before sedimentation of the precipitated protein had occurred. B. Tube showing re-solution of precipitated macroglobulin as a result of the addition of a solution of sodium chloride.





Skin lesions in cryoglobulinaemia A Areas of skin necrosis on the lobe of the ear of a patient with cryoglobulinaemia B Purpura and dry gangrene on the foot of the same patient

PLATE II



A Tube containing serum from a patient with cryoglobulinaemia. The serum has been kept at 4° C for 24 hr. The cryoglobulin has come out of solution and can be seen as a sediment at the bottom of the tube. B Cellular aggregation ("sludging") in blood in the conjunctival vessels in a patient with multiple myelomatosis who had a high concentration of abnormal proteins in the plasma. (Reproduced from Marmont *et al* (1957) by permission of *Acta Haematologica*.)





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Although a few cases have been described in which the cryoglobulinaemia was apparently not due to any pre existing disease (Gunz 1956 Mackay *et al* 1956 Volpe Bruce Robertson Fletcher & Charles 1956) the majority of cases have had some form of reticulosis particularly myelomatosis lymphosarcoma lymphatic leukaemia or lymphadenoma Cryoglobulinaemia has also been observed in chronic inflammatory conditions such as rheumatoid arthritis and kala azar

Cryoglobulinaemia is sometimes associated with purpura which occurs first and most characteristically in areas exposed to the cold the ears the tip of the nose the hands and the feet Purpura also occurs however in areas not so exposed The purpuric areas of skin which may be painful (Gunz 1956) frequently undergo necrotic changes Typical pictures of the skin in cryoglobulinaemia are shown in Plate 10

An atypical form of Raynaud s syndrome is sometimes seen and some patients with cryoglobulinaemia develop cold urticaria (Waldenstrom 1952*b* Gunz 1956 Stefanini & Dameshek 1955)

Owing to the fact that many cryoglobulins come out of solution at room temperature and often form a gel at that temperature (Stefanini & Dameshek 1955) the erythrocyte sedimentation rate taken at room temperature may give a falsely low reading When taken at 37 C the sedimentation rate is invariably raised

Diagnosis is simple Blood should be kept at 37 C until the serum has separated The serum should then be transferred to a second tube and kept at 4 C The cryoglobulin will come out of solution and will readily redissolve on warming Plate 11 (A) shows a tube of cryoglobulin containing serum which has been kept for 24 hr at 4 C The cryoglobulin has come out of solution and can be seen as a compact mass at the bottom of the tube Sometimes as I have said the cryoglobulin forms a gel on cooling in which case sedimentation of the abnormal protein does not occur but instead the serum becomes transformed into a solid gel which again becomes fluid on warming

Causes of abnormal bleeding in the dysproteinaemias

The bleeding time and capillary fragility have often been noted to be increased in cases of abnormal bleeding associated with dysproteinaemia (Stefanini & Dameshek 1955) It is clear therefore that bleeding in these conditions is predominantly of the capillary type

The cause of the bleeding in hyperglobulinaemic purpura is unknown Waldenstrom (1952*a*) has suggested that it may be due to a deficiency in some globulin fraction necessary for the maintenance of a normal capillary resistance

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In primary amyloidosis, purpura is probably due to infiltration of vessel walls with amyloid substance (Propp *et al* 1954, Stefanini & Dameshek 1955)

In secondary amyloidosis, myelomatosis macroglobulinaemia and cryo globulinaemia, bleeding is probably due to a combination of factors varying in significance from case to case Of these the following appear to be the most important

Thrombocytopenia This is common in myelomatosis and in the reticu loses which appear to be the cause of many cases of secondary macro globulinaemia and cryoglobulinaemia Thrombocytopenia is probably the most important single factor predisposing to abnormal bleeding in many patients with dysproteinaemia

Coagulation defects A variety of coagulation defects have been described although not all cases with such defects have suffered from abnormal bleeding Long and his colleagues (1955) have described a case of malignant lymphoma associated with macroglobulinaemia in which the abnormal protein which had a sedimentation constant of 19 Svedberg units interfered with the activity of factors v and vii In interesting contrast to this, Jim & Steinkamp (1956) have reported a case (see below) in which there was factor v deficiency but in which the abnormal globulin did not interfere with the action of normal factor v The major component of the abnormal globulin in this case had a sedimentation constant of 13 9 Svedberg units The diagnosis was obscure Although the authors suggested that it was a case of Waldenstrom s macroglobulinaemia the sedimentation constant of the protein was too low, and their alternative diagnoses of myeloma or lymphocytic leukaemia seem more probable The diagnosis in the case reported by Voigt & Frick (1956) was also obscure Although they describe it as macroglobulinaemia of Waldenstrom the sedimentation constant of the abnormal protein was only 12 6 Svedberg units This case was deficient in prothrombin and showed defective conversion of prothrombin to thrombin These workers did not however, investigate the possibility that this latter abnormality might have been due to the presence of the abnormal protein In a case of cryoglobulinaemia associated with hepatic cirrhosis, in which the abnormal protein contained a high concentration of macroglobulins (sedimentation constants 19 Sved berg units and over) the platelets showed diminished formation of pseudo podia and their capacity for adhering to foreign surfaces was reduced (Braunsteiner Falkner, Neumayer & Pakesch 1954) These abnormalities appeared to be due to an action of the abnormal proteins

In some cases thrombin formation has been normal but there has been impaired conversion of fibrinogen to fibrin even in the presence of added

thrombin Ratnoff (1953) has described several cases of myelomatosis in which this was observed He was able to show that the delayed conversion of fibrinogen was due to a factor in the patients plasma He did not report the sedimentation constants of the serum proteins in his cases Sometimes the impaired conversion of fibrinogen to fibrin has been shown to be due to the action of the abnormal protein Such cases have been described by Luscher & Labhart (1949) Uehlinger (1949) and by Craddock Adams & Figueroa (1953) The latter authors although they were unable to isolate the abnormal protein from the patient s plasma showed that an apparently identical protein in his urine inhibited fibrin formation in normal plasma The patient s plasma and serum had the same effect The abnormalities in the three cases reported by these three groups of workers appeared to be the same In each the primary condition was myelomatosis In the only case in which the sedimentation constants of the serum proteins were measured they were found to be normal (Uehlinger 1949) In each the blood formed a gel when allowed to clot but the gel contained no fibrin strands Plasma treated with thrombin developed a similar fibrin free gel Fibrin formation did however occur if the plasma was diluted with normal saline Craddock Adams & Figueroa (1953) found that the addition of calcium chloride solution caused fibrin formation in plasma from their patient although the calcium concentration in his plasma was normal As calcium is necessary for normal fibrin formation these workers suggested that the abnormal protein might have an affinity for calcium and that in consequence there was inadequate calcium available to permit fibrin formation This hypothesis however leaves unexplained the fact that thrombin formation which also requires calcium was not deficient in this patient

Finally in the case referred to above Jim & Steinkamp (1956) have shown that in addition to the deficiency of factor v the conversion of fibrinogen to fibrin was abnormal although the abnormal protein did not interfere with the conversion of normal fibrinogen In this case the patient s own fibrinogen was abnormal and even when isolated from her other plasma proteins was not converted normally to fibrin in the presence of thrombin

To summarize it may be stated that a number of different coagulation defects have been described One—defective conversion of fibrinogen to fibrin—is sometimes due to the action of the abnormal protein although the mechanism by which it produces this effect is not known Insufficient work has been done on this subject to make it possible to say whether any particular type of dysproteinaemia is more liable than another to be associated with a particular coagulation defect

Infiltration of blood-vessel walls Actual infiltration of blood vessel walls

by amyloid has been observed in both primary and secondary amyloidosis and appears to have caused extensive purpura (see Propp *et al* 1954) Stefanini & Dameshek (1955) consider that infiltration of vessel walls with other abnormal proteins in the dysproteinaemias may be a factor predisposing to bleeding

Intravascular cellular aggregation Plate 11 (B) shows the effect of myeloma protein on circulating blood cells in the conjunctival vessels This phenomenon of cellular aggregation in circulating blood is reflected in the rouleaux formation seen, for instance during determination of the very high erythrocyte sedimentation rate It is known as sludging The cellular aggregations retard the capillary circulation and at times temporarily arrest the flow of blood (Marmont Fusco Gay & Mariotti 1957) It is difficult to avoid the conclusion that this will result in some degree of capillary anoxia which may increase capillary fragility and so contribute to the haemorrhagic tendency

In macroglobulinaemia sludging may well be of more importance than in myelomatosis because owing to the presence of the macroglobulins the plasma viscosity is greatly increased This may itself slow blood flow and so *increase the effects of sludging' in causing capillary anoxia*

In cryoglobulinaemia sludging' is almost certainly even more important In a case of myelomatosis with cryoglobulinaemia Barr Reader & Wheeler (1950) found that cooling the conjunctival vessels caused a marked increase in 'sludging This effect is not seen in cases of myelomatosis without cryoglobulinaemia (Marmont *et al* 1957) The plasma viscosity rises when cryoglobulinaemic blood is cooled This is shown by the fall in the sedimentation rate which occurs on cooling Furthermore Gunz (1956) has shown that cryoglobulins can begin to come out of solution *in vitro* at a temperature as high as 35 C This is a temperature that must often be *reached in vessels in areas exposed to the cold* In consequence it seems almost certain that actual precipitation of cryoglobulin must occur in such vessels It seems probable therefore that the increase in sludging the increase in viscosity and the protein precipitation that occur on cooling must produce capillary anoxia and so tend to cause spontaneous bleeding Protein precipitation is almost certainly also the cause of the necrotic lesions seen in areas of skin which have been exposed to cold

Hepatic and renal failure If the primary disease causes extensive hepatic or renal damage there may be a reduction in the level of various clotting factors in the blood as a result of hepatic involvement or uraemic vascular damage as a result of involvement of the kidneys Such changes may increase the bleeding tendency or may be the main abnormality causing bleeding in some patients with dysproteinaemia

To summarize the haemorrhagic diathesis seen in some cases of dysproteinaemia is ill understood The cause of the purpura in hyperglobulinaemic purpura is not known In primary amyloidosis purpura is probably due to infiltration of vessel walls with amyloid substance In secondary amyloidosis myelomatosis macroglobulinaemia and cryoglobulinaemia several factors are involved

1 Thrombocytopenia when present

2 Coagulation defects including abnormal conversion of fibrinogen to fibrin in the presence of thrombin

3 Infiltration of vessel walls by amyloid or other abnormal proteins

4 Sludging which by slowing or even temporarily arresting capillary circulation may produce capillary anoxia and so increase capillary fragility This is probably of most importance in cryoglobulinaemia in which condition sludging is markedly increased by cooling which also increases the viscosity of the blood and causes protein precipitation—all factors which must impair capillary circulation Intravascular protein precipitation is almost certainly a major factor causing the necrotic skin lesions seen in this condition in areas exposed to cold

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